University of Chester

Development, Digestibility and Oxidation Properties of LC3PUFA Nanoemulsion and Its Effects on Sensory Profile of Food

Thesis submitted in accordance with the requirements of the University of Chester for the degree of Doctor of Philosophy

By

QiQian Zhou

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Table of Contents

List of tables

List of Figures

Abstract

Development, digestibility and oxidation properties of LC3PUFA oil nanoemulsion and its effects on sensory profile of foods

Qiqian Zhou

The long chain omega-3 polyunsaturated fatty acids (LC3PUFA) in human diets are mainly derived from oily fish and fish oil based supplements. Currently, the consumption of oily fish in the UK is far below the recommended level. LC3PUFA's non-fish sources such as algal oil with DHA (docosahexaenoic acid) are particularly important for vegetarians, non-fish eaters, and pregnant women. In previous work, high DHA vegetative algal oil load 50% w/w was successfully used to develop an oil-in-water nanoemulsion system suitable for functional food enrichment.

The aims of this study included to investigate the effect of selected emulsifiers on oil-in-water nanoemulsions of algal oil prepared using ultrasonic technology. To improve the stability and digestibility of nanoemulsions within an *In vitro* digestion model. To examine the oxidation stability of nanoemulsions of algal oil and bulk algal oil with composition and droplet size changes during a 5 weeks storage trial at a temperature of 4 °C, 20 °C and 40 °C respectively. To evaluate sensory properties and consumer acceptability of food products with the incorporation of resulted nanoemulsion and find out possible relationship between the sensory profile of foods and the characteristics of added nanoemulsion.

Nanoemulsion of LC3PUFA algal oil was developed with selected 6% w/w emulsifiers, including Lecithin (LN), Tween 40 (TN), Tween 60, equal ratio of Tween 40 and lecithin (LTN), 50% w/w Algal oil and 44%w/w water using a homogenizer and ultrasound processor. The results show that the nanoemulsion has been stabilised with selected emulsifiers (LN, TN & LTN) and the smallest droplet size of nanoemulsion was obtained using the combination of lecithin and Tween 40 at ratio 50:50.

The *In vitro* digestion experiments were conducted with a model of fed state gastric and duodenal digestion using method of Lin *et al* (2014). The results show that the omega-3 oil nanoemulsion (LE/TW 50:50) were stable over 60 min in the gastric phase, in contrast omega-3 nanoemulsion (LE 100%) was destabilised at the gastric phase in 60 min, in which the droplet size diameter was significantly larger than at the beginning of gastric phase ($P \leq 0.05$).

The droplet size, fatty composition and oxidised compounds were measured to compare bulk algal oil and nanoemulsions stabilised with lecithin (LN) and Tween 40 (TN) solely and in combination (LTN) over a storage period of 5 weeks at temperatures of 4, 20 and 40°C. The results show the droplet size of nanoemulsions had no significant changes for samples stored at tested temperatures over 5 weeks storage. There were no significant differences in DHA composition within the weeks and temperatures used. For the GCHS analysed results, the increase in temperature to 40 ºC and storage time had a significant effect on the development of propanal for all samples ($P \leq 0.05$). Nanoemulsions prepared with lecithin alone had significantly higher development of propanal in week 1 at both 40 ºC and 20 ºC ($P \leq 0.05$). Lecithin (sole and combination with Tween 40) had more significant increases in oxidised volatiles at 40°C, which may be due to the instability of linoleic acid found in lecithin molecules which located in the outer layer of the oil droplets. There were no significant increase in oxidised compounds from the beginning to the end of storage for all tested samples stored at 4 °C.

The sensory testing was also conducted on white sauce incorporated with omega-3 nanoemulsions with selected emulsifiers and bulk algal oil. The results show that the sensory attributes and overall acceptability of foods enriched with omega-3 nanoemulsion were statistically significantly lower than that of control sample ($P \leq 0.05$).

Overall, the smallest droplet size of nanoemulsion was achieved with combination of lecithin and Tween 40 at a ratio of 50:50 by using ultrasonic processor. The stability and digestibility of nanoemulsion with the combination of lecithin and Tween 40 was improved in an *In vitro* digestion approach. A storage period of 5 weeks and temperature have no significant effect on the droplet size of tested nanoemulsion samples. However, there is a significant increase of the oxidised volatiles at 40 °C for all samples. Sensory testing show the white sauce with nanoemulsion has a stronger fishy taste and less overall liking than with bulk oil, indicating the smaller drop size is more ready to spread and reach the sensors of the mouth.

Key Words: LC3PUFA, Omega-3, Nanoemulsion, Algal oil, lecithin, Tween 40, Stability, Oxidation, Bioavailability, Sensory, droplet size

Chapter 1 Introduction

1.1 Background

The long chain omega-3 (n-3) polyunsaturated fatty acids (LC3PUFA) in human diets are mainly derived from oily fish, and fish oil based supplements (Lenihan-Geels & Bishop, 2016). At present, the consumption of oily fish in the UK is far below the recommended level which is 0.2 g per day (Bates *et al*., 2016). LC3PUFA's non-fish sources such as algal oil are particularly important for vegetarians, non-fish eaters, and pregnant women (Ryan & Symington, 2015). Algal oil has recently become a sustainable vegetarian source of long chain omega 3 fatty acids long chain omega 3 fatty acids (Solans & Solé 2012).

The current delivery system of nutrients in the market could be further improved by using nanoemulsion technology instead of just simply enriching with micronutrients to enhance the nutritional properties. Omega-3 fatty acids are a set of polyunsaturated fatty acids, found in deep-sea fishes and certain plants that are beneficial to human health (Meyer *et al.*, 2003). The previous studies showed that nanoemulsion of enriched nutrients had significantly negative effects on sensory attributes of food (Lane, 2013), and demonstrated the sensory characteristics varied with emulsifiers and drop sizes of emulsion (Van Ruth *et al*., 2002).

In previous work, high DHA vegetative algal oil load of 50% w / w was successfully used to establish an oil-in-water nanoemulsion system suitable for functional food enrichment. (Sprague *et al*., 2017) Nanoemulsions systems having a droplet size in the range of 20-500 nm that can improve LC3PUFA bioavailability through ultrasound processing (Lane *et al.*, 2014). However, the application of ultrasound may also affect the oxidation stability of LC3PUFA (Pingret *et al*., 2013).

1.2 Aims

1. To investigate the effect of selected emulsifiers on oil-in-water nanoemulsions of algal oil prepared using ultrasonic technology.
2. To test the absorption and stabilizing properties of polysaccharides for an outer layer out of a nanoemulsion.
3. To improve the stability and digestibility of nanoemulsions prepared in an *In vitro* digestion model.
4. To examine the oxidation stability of nanoemulsions of algal oil and bulk algal oil with composition an droplet size changes during an 5 weeks storage trial at a temperature of 4 °C, 20 °C and 40 °C respectively.
5. To evaluate sensory properties and consumer acceptability of food products with the incorporation of resulted nanoemulsion and find out possible relationship between the sensory profile of foods and the characteristics of added nanoemulsion.

Chapter 2 Literature review

2.1 The application of nanoemulsion technology

A nanoemulsion is defined as a discrete entity and the size of each droplet can be measured in a scale of mean radius from 10 to 100 nm (Tadros *et al*., 2004). In comparison to an ordinary emulsion, nanoemulsions are more stable to gravitational separation, flocculation and coalescences (McClements, 2011). The current delivery system of nutrients in the market could be improved further by using nanoemulsion technology instead of just simply enriching with micronutrients to enhance nutritional properties.

The relationship between diet and health has received an increased attention in recent years around the world. For this reason, the demand for functional foods is growing steadily (Sagalowicz & Leser, 2010). The reason being is that some of these nutrients are insoluble in water, showing a restricted and uncontrolled bioavailability (Acosta, 2009). The delivery systems of nanoemulsion have been developing in recent years (McClements, 2011). An advantage of using nanoemulsion instead of an ordinary emulsion delivery system is the fact that the bioavailability and bioefficacy of the delivered lipophilic bioactive compound can be expected to be increased when delivered in the form of a nanoemulsion (Sagalowicz & Leser, 2010).

2.2 Composition of nanoemulsion

2.2.1 Emulsifiers

The emulsifier is one of the most important factors to be considered when designing a nanoemulsion. Emulsifiers are surface-active substances, which can be adsorbed at the oil-water interface and prevent the droplet in the dispersed phase in an emulsion from

aggregating (Jafri *et al.*, 2007). This methodology is commonly used in food and pharmaceutical industries. Emulsifiers can easily be adsorbed at the surface of the droplet mobile phase interface in order to reduce the interfacial tension and prevent the process of homogenization by droplet coalescence (McClements, 2005; Gambi & Grosso, 2011). Different emulsifiers have different structures and functions and the influences on emulsion stability according to their physical and chemical properties (Stang *et al*., 2001).

The small molecule surfactants, phospholipids, proteins and polysaccharides are used as emulsifiers in food industry (McClements, 2011). The small molecule surfactants are classified according their electrical characteristics, such as, ionic, non-ionic, and zwitterionic (McClements, 2005). The different types of emulsifiers require different methods to create nanoemulsions, for example, proteins and polysaccharides have the advantage of being natural food ingredients (McClements, 2011),

Therefore, choosing a suitable emulsifier is one of the key issues in the production of emulsions and dispersions. Emulsifiers can stabilize emulsions and dispersions by electrostatic and / or steric hindrance mechanisms (Tan & Nakajima, 2001). Non-ionic emulsifiers do not affect the static stability, but they are very good steric stabilizers. In most cases, these emulsifiers are insensitive to the presence of any ionic solution; therefore, they are widely used in various nanoemulsion systems, in particular when the hardness of water is not identified. Some emulsifiers are hydrophilic, they therefore repel each other, thereby providing stable emulsions and dispersions (McClements, 2005).

Determination of whether an emulsifier is hydrophilic can be made using a hydrophilic lipophilic balance value (HLB) as shown in Figure 1; high HLB values indicate a more hydrophilic emulsifier. Emulsifiers having an HLB value between 3.5 and 6 are more suitable

for water in oil (W / O) emulsions, and those having an HLB value between 8 and 18 are more suitable oil in water (O / W) emulsions (Tan & Nakajima, 2005a). Most commercial emulsifiers, especially sucrose esters, mono-, di- and tri-esters, are mixtures. The HLB value of a mixed emulsifier depends largely on the monoester content; a higher monoester content gives higher HLB values. HLB value which is ascribed to an emulsifier depends on the fatty acid chain length (S); short chain fatty acids result in a higher HLB value (Whitehurst, 2004).

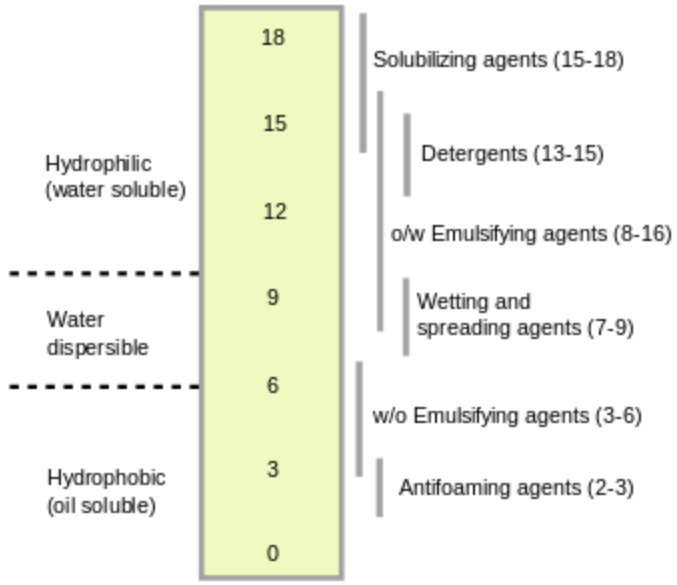

Figure 1 The list of HLB (Aulton, 2002)

2.2.1.1 Lecithin

Lecithin was first found in egg yolk by the French chemist Maurice Gobley in 1846. He also isolated the same lecithin from brain, bladder and other materials from animals. The important characteristic of these materials is that they contain phosphorus, organically bounds to a lipid-type structure (Whitehurst, 2004). Lecithin can be obtained from vegetable sources, which are manufactured exclusively as by-products of the vegetable oil refining process (Belayneh *et al*., 2018. Naue *et al*., 2018). The other extraction processes have been developed and are applied commercially (Whitehurst, 2004).

Lecithin is a basic substance of life, which is not only important for biofilm in the human body, but also a source of choline and fatty acids (Rydhag & Wilton, 1981). It is needed to maintain the normal metabolism and physiological activity of biofilms in the body, which was hailed as "vascular scavenger". Lecithin is a full-featured nutrient, beneficial to the prevention and improvement of cardiovascular disease, cerebrovascular disease and brain. It also prevents fatty liver and cirrhosis, as well as skin enhancement (Whitehurst, 2004).

As an emulsifier, it is known as phospholipids. As Figure 2 shows they have a polar and a non-polar end, which enables them to act as barriers between aqueous solutions and non-aqueous solutions. The molecules of phospholipids line up in a way that their polar “head”, which composed of glycerol and fatty acid end oriented toward the water molecules. The non-polar tails, which consists of a long fatty acid tend to orientate away from the water. In other words, the polar ‘head’ is hydrophilic or water loving and the non-polar “tail” is hydrophobic or non-water loving in nature (Shaw *et al*., 2007, Klikesorn *et al*., 2005).

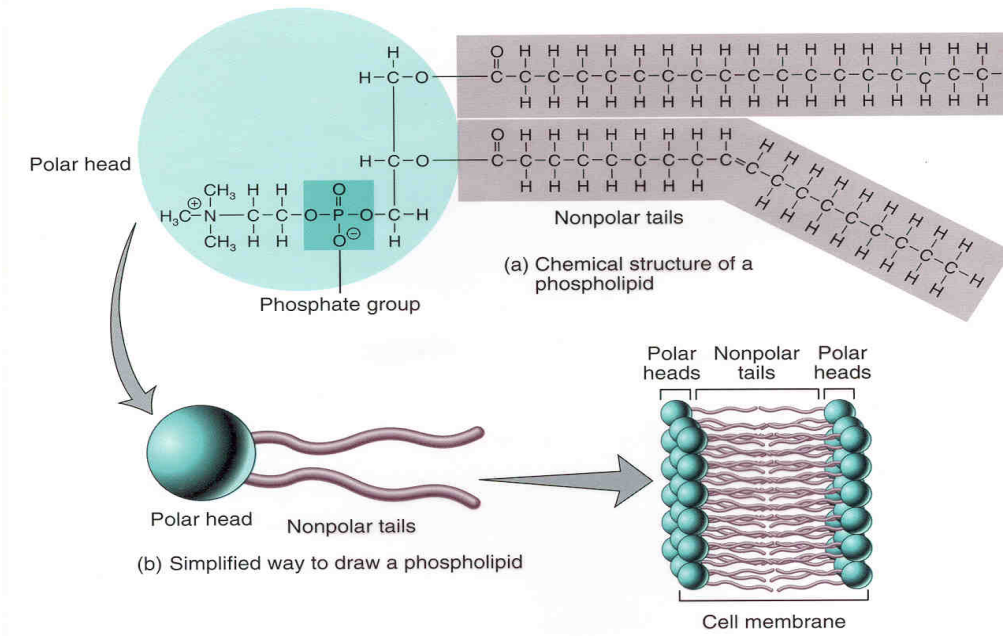

Figure 2. The structure of lecithin (Phospholipids Analysis Service, 2018).

Hence, when phospholipids are placed in an aqueous environment, spontaneously the hydrophobic portions stick together, as do the hydrophilic and produce a very stable form. This arrangement of molecules is called as lipid bilayer or phospholipid bilayer. The spontaneous arrangement of phospholipid molecules into a bilayer creates the lowest free-energy configuration in which the hydrophobic regions move away from water, while the hydrophilic regions interact with the water (Klikesorn *et al*., 2005; Shaw *et al*., 2007).

2.2.1.2 Tweens

During 1942, 'Tween' was registered as the formal trading name for polyoxyethylene (20) sorbitan ester or polysorbate. They are amongst the most universally recognized, safe, regulatory approved, high performance emulsifiers used in wide aspects such as food industries, personal care, cosmetic, textile and pharmaceutical industries (Whitehurst, 2004). The polysorbate is equivalent to the sorbitan ester having a molecular weight of about 20 moles of ethylene oxide manufacture. This is due to the presence of long chain polyoxyethylene obtained, and the most hydrophilic nonionic emulsifier (Anarjan & Tan, 2013).

Sucrose fatty acid esters are sucrose, which contain three primary hydroxyl groups and five secondary hydroxyl groups esterified eight synthetic polyols, as Figure 3. shows; primary group is more reactive and more easily replaced with a fatty acid. Each distinctive features of the polysorbate or sucrose esters of fatty acids has a variety of uses in its structure. In nutritional understanding, they are digested subsequent enzymatic hydrolysis of the molecule, which can be metabolized in the usual manner of components (Whitehurst, 2004).

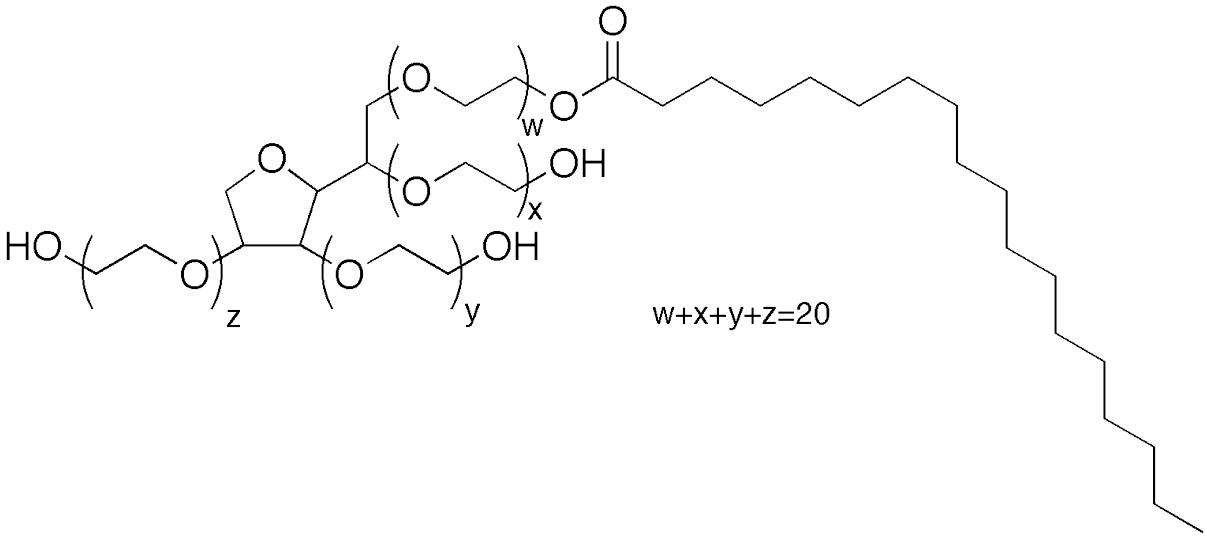

Figure 3 The structure of Tween 40 $C_{62}H_{122}O_{26}$ (Sannaningannavar *et al.,* 2014)

In a previous work (Anarjan *et al*., 2010), the effect of emulsification and evaporation parameters on the physicochemical properties of astaxanthin nanodispersions was investigated, and the optimum processing conditions were obtained. It was shown that the emulsification/solvent evaporation technique was applicable for the preparation of astaxanthin nanodispersions. However, in that study, Polysorbate 20 was the only small-molecule, nonionic emulsifier used to stabilize the nanodispersions.

2.3 The development of nanoemulsion

In the past few years, nanotechnology had become one of the most interesting areas of scientific research (Beck *et al.*, 2009). In the field of food science, the properties of nano materials have been extensively investigated and are well understood, allowing scientists to develop new, healthier, tastier and safer food by using nanomaterials (Tan & Nakajima, 2005b).

In order to create a stable nanoemulsion, there are a number of different approaches, such as high-energy or low-energy approaches depending on the underlying principle (Tadros *et al*.,

2004; Acosta. 2009; Anton & Vandamme, 2009). High-energy approaches are the most common method used to prepare nanoemulsion; examples are high-pressure valve homogenizers, micro-fluidizers and sonication methods (Gutierrez *et al*., 2008; Velikov *et al*., 2008).

An ultrasonic homogenizer is a very effective method of generating and processing nano-size materials (Leong *et al.*, 2009). In general, a liquid ultrasonic cavitation can cause rapid and complete degassing: by generating free chemical ions (radicals) lead to a variety of chemical reactions accelerate chemical reactions by facilitating the mixing of reactants. In addition, enhancing polymerization, depolymerisation the reaction product temporarily or permanently breaks aggregates dispersed polymer chain chemical bonds; improve emulsification rate; increase diffusion rate; homogeneous emulsion or micronized or nano sized material to produce highly concentrated dispersion (Strawbridge *et al*., 1995; McClements, 2005).

As water solubility improve, a variety of functional lipid bioactive compounds, such as carotenoids, plant sterols, polyunsaturated fatty acids and many other compounds were used to enrich with food (Tan & Nakajima, 2005b; Beck *et al*., 2009). The increased bioavailability of nutrients in food is part of the reason why nanotechnology in food is an important application. These functional lipid compounds and nano-technology application have received particular interest in the past few years in food, pharmaceutical and cosmetic industries due to the enhance benefits and cost effectiveness (Leong *et al*., 2011).

2.4 Omega-3 fatty acid oil

2.4.1 Omega-3 fatty acids and Algal oil

Omega-3 fatty acids have received a lot of attention in the research community recently due to the potential health benefits identified in the epidemiological studies of Greenland's Inuit

population by Dyerberg *et al*. (1975). Research demonstrated a positive relationship between the intakes of LC3PUFA and reduced cardiovascular disease risk (Murphy *et al*., 2007) as well as important functions in brain health (Breivik, 2007). The formal acknowledgments of the importance were made to long chain omega-3 polyunsaturated fatty acids in a number of national and international groups (Barrow *et al*., 2008).

Excess omega-6 fatty acids stimulates the formation of arachidonic acid (ARA), the fatty acid precursor of prostaglandins and other eicosanoids that are involved in inflammation (Raper, N. R., Cronin, F. J., & Exler, J., 1992). Numerous studies have shown that there has been a significant decrease in the dietary intake of n-3 PUFA along with a substantial increase in the consumption of n-6 PUFA in Western populations (World Health Organization, 1993, The British Nutrition Foundation, 1992). It was indicated health benefits were validated in the ratio of n-3 to n-6 fatty acids intake is estimated to be 1 in 20 respectively in a modern western diet (DeFilippis & Sperling, 2005) when compared to the Palaeolithic's diet which consisted of a 1 in 2 ratios. Also in the US, the present ratio is greater than 10:1, causing a deficiency of the omega-3 fatty acids (Raper, N. R., Cronin, F. J., & Exler, J., 1992).

It was suggested that humans could convert omega 3 and 6 to more physiologically active fatty acids by elongation and desaturation. The long chain fatty acids are produced in the metabolic pathway of n-6 and n-3, which compete for the enzymes for conversion and it showed that conversion of n-6 is more efficient than n-3 (Davis & Kris-Etherton 2003).

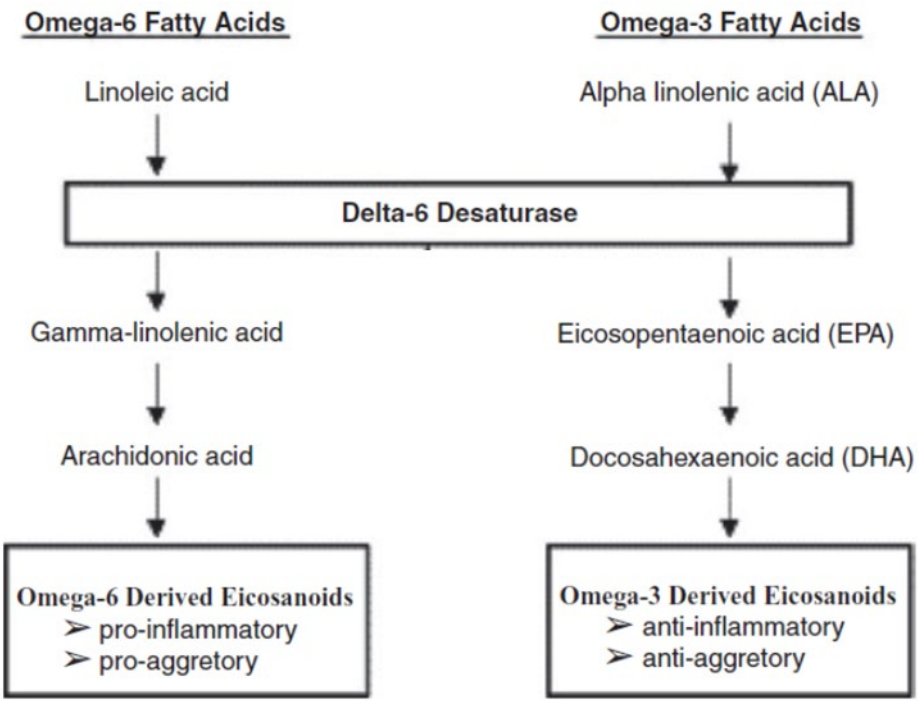

Figure 4 Metabolic pathway of omega-6 and omega-3 fatty acids. (Defilippis and Sperling, 2005).

The main cause of the occurrence was suspected to be the restricted consumption of fish. It was reported that fish and fish oils are main sources of long-chain omega-3 polyunsaturated fatty acids (Davis & Kris-Etherton, 2003). Plants such as nuts and seeds are also rich in omega-3 (DeFilippis & Sperling, 2005). α-linolenic acid (ALA) is the main omega-3 in vegetarian source and ALA commodities are particularly rich in flaxseed, walnuts, and soy. Consuming a diet, that is low in n-3 and high in n-6 may induce health problem due to the differences in metabolic pathways.

The recommendation of fish consumption stated that general population should consume eat two portions of fish, in which one has to be oily fish on a weekly basis in the UK that is equitant to 0.2 g/day long chain omega-3 polyunsaturated fatty acids (SACN 2004). Under consumption of fish is the common occurrence in the UK population with just around 20 g/week (SACN 2004). The use of supplement may be another source to reach recommended

level of omega-3 but only 11% of male and 13% of women were found to take fish oil supplement, according to the National Diet and Nutrition Survey (2011). A relatively low consumption of long-chain omega-3 polyunsaturated fatty acids was identified in vegetarians when compared to omega-6 intakes and decreased body storage of EPA and DHA were the highest amongst vegetarian population (Davis and Kris-Etherton 2003).

There are no official n-3 recommendations for vegetarians and vegans in present but it was advised that vegetarians should consume a double amount of recommended intake of ALA (Davis and Kris-Etherton 2003). In addition, pregnant and breastfeeding mothers are among the group that required a high consumption of L long chain omega-3 polyunsaturated fatty acids needs (Derbyshire 2009, SACN 2004). It found that the foetus accumulates 60 to 70 mg LC3PUFA daily during the last trimester of pregnancy, therefore pregnant women are recommended to consume three times more of the daily recommended intake of 100 mg DHA for an adult (SACN 2004). The oily fish consumption was proposed to be a worrying factor due to the environmental contaminants and sustainable food supply (Derbyshire 2009).

DHA is the major fatty acid component which has been found from brain contain 60% lipid. DHA is important in brain and retinal development and its store are frequently deficient at birth in the preterm infant (The British Nutrition Foundation, 1992).

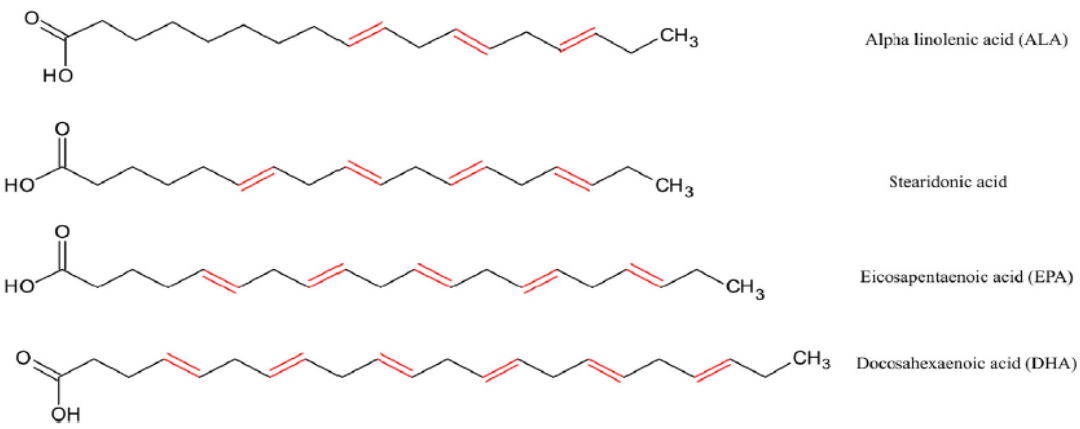

Figure 5 Chemical structure of omega-3 fatty acids

DHA is also required for maintenance of normal brain function in adults. Decreases in DHA in the brain are associated with cognitive decline during aging and with the onset of sporadic Alzheimer disease (FAO/WHO. 1993). The omega-3 fatty acids from vegetable origin extracted are found from echium seed oil, chia seeds, camelina, perilla, flax seeds (Comunian *et al.*, 2016), and algal (Breivik, 2007).

Table 1 Omega-3 content of vegetarian source

Source	ALA %	EPA%	DHA%	Reference
Algal	-	0.29	46	Breivik, 2007
Echium seed	33	-	-	Berti *et al*. 2008
Flax seed	57	-	-	Sultana, 1996
Chia seeds	56.9-64.8	-	-	Ayerza & Coates, 2011
Camelina	36	-	-	Sultana, 1996
Perilla	53	-	-	Sultana, 1996

Algal oil are organisms ranging in size from 0.2 to 2.0 µm in diameter (microalgal) up to 60 m length (macroalgal or seaweeds). Microalgal is now being used in biotechnology and already commercialized that belong to the green algal (Christaki *et al*., 2013). They contain many valuable components, such as polyunsaturated fatty acids, tocopherols, and sterols, vitamins and minerals.

Algal oil is created in controlled and closed fermentation facilities that are purely vegetarian (Breivik 2007). This is a novel material to be used in the food industry. Derbyshire (2009) state that functional foods and supplements from algal derived DHA is a great solution to tackle the low consumption of fish. It also indicated that more investigation should be conducted to evaluate the effectiveness of algal oil in order to support the use of it for food fortification in the future.

Production of docosahexaenoic acid (DHA; 22:603) from algal oil was achieved using urea complexation technique. The major fatty acids found in algal oil were 22.6, 14:0 and 18:1. As a result of urea complexation, 14.0, 16:0 and 18:1 were eliminated almost completely while DHA was enriched from 47.4 to 97.1% with a process yield of 32.5% of the weight of the original algal oil. The recovery efficiency of DHA was considerably higher and 66.5% of the total DNA present in the original algal oil was found in the DHA concentrate following the urea complexation process (Senanayake S. N. & Shahjdi F., 2000).

Omega-3 fatty acids have received a lot of attention in the research community recently due to the potential health benefits identified in the epidemiological studies of Greenland's Inuit population by Dyerberg et al. in 1970. Research demonstrated a positive relationship between the intakes of LC3PUFA and reduced cardiovascular disease risk (Murphy *et al*., 2007) as well as important functions in brain health (Breivik, 2007). The formal acknowledgements of

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2.5 Definition of Bioavailability and digestibility

2.5.1 Bioavailability

'The rate and extent to which the active ingredient (in this study omega-3) is absorbed and become available at the site of action' defined by the food & Drug Administration (2003). And also a study (Huang *et al*., 2004) state that 'the function of an ingested component (or its product) that eventually ends up in the systemic circulation.

2.5.2 Digestibility

'The quantity of food that the body retains after a meal. Mathematically, it is mass of food consume.' defined by medical dictionary (2009). Also, defined by dictionary of food and nutrition (Oxford university press, 2005) which is 'The proportion of a food stuff absorbed from the digestive tract into the blood stream, normally 90-95%.'

2.5.3 *In vitro* Digestion Model

In vitro digestion models are widely to study the structural changes, digestibility, and release of food components under simulated gastrointestinal conditions. In principle, *in vitro* digestion model provide a useful alternative to animal and human model by rapidly screening food ingredients. The ideal *in vitro* digestion method would provide accurate results in a short time and could thus serve as a tool for rapid screening food or delivery system with different compositions (Coles, Moughan, & Darragh, 2005). Most frequently utilised enzyme and other biological molecule used within *in vitro* digestion models were pepsin, pancreatin, trypsin, chymotrypsin, peptidase, α-amylase, lipase, bile salt, and mucin. The types of enzyme including within an *in vitro* digestion model tend to reflect the major food component being investigated, e.g., lipase for lipid digestion, proteases for protein digestion, and

amylases for starch digestion (Green *et al*., 2007, Bering *et al.*, 2006, Bravo *et al.*, 1998, Versantvoort *et al*., 2005).

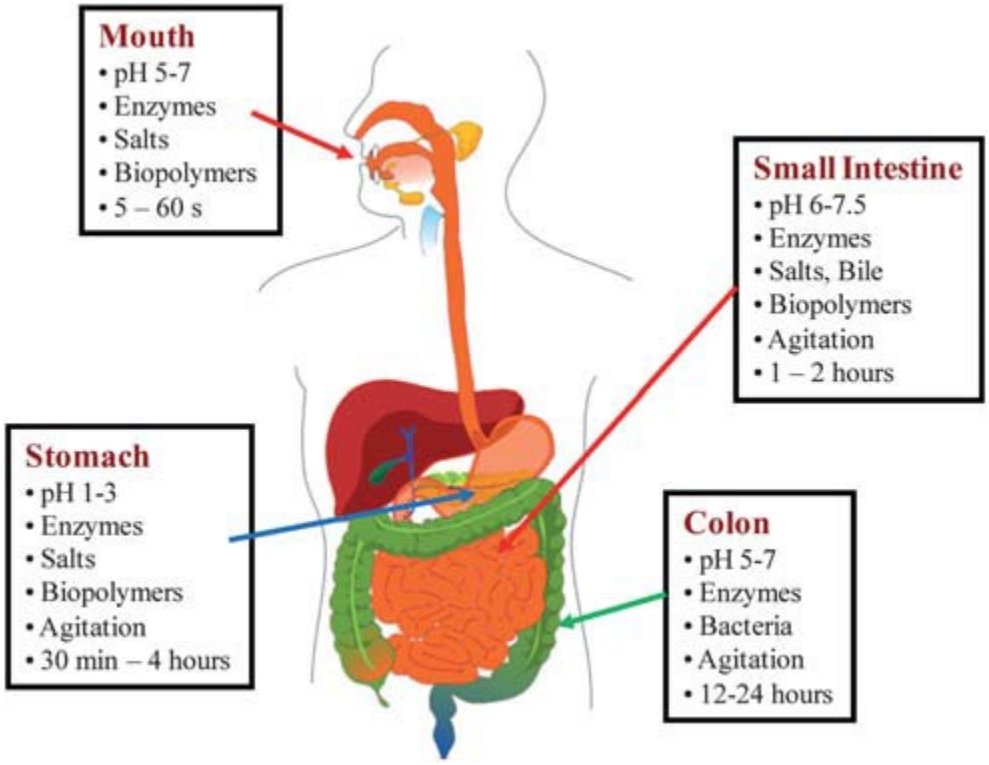

Figure 6 Schematic diagram of the physicochemical conditions in the different regions of the human GI tract (McClements & Li, 2010).

The figure 6 shows the human digestion system. There are three tips need to be noted to design an *in vitro* digestion model. Firstly, different enzymes are normally adding separately rather than together, because of simulate the different stages during the digestive process (Boisen & Eggum, 1991). Secondly, enzymes often operate with additional component in digestive fluids, such as pancreatic lipase requires operate with calcium and bile salts (Boisen & Eggum, 1991). Finally, the digestive fluids are important to prepare freshly for each study, because of the enzyme activity may decrease over time (S.J. Hur *et al*., 2011). In addition, the digestion temperature is 37 °C to help the enzyme digest the food samples. The range of digestion time for test sample incubation in simulated stomach, small intestine, and large

intestine fluids. The *in vitro* digestion time must be considered the particle size of the test sample contain, and also, the enzyme concentration is very important factor to consider when designing an *in vitro* digestion model (S.J. Hur *et al*., 2011).

In this study, the main enzymes being using to lipid digestion, which are pancreatic lipase and bile salt. Digestion and transport of lipids poses unique problems relating to the insolubility of lipids to water, because lipids must be transported through aqueous compartments within the cell as well as in the blood and tissue spaces (Boisen & Eggum, 1991).

Bile salt are polar derivatives of cholesterol, formed in liver and secreted into gall bladder. Its pass via the bile duct into the intestine. Bile salts emulsify with phospholipids as vehicle for fat globules into smaller micelles, increasing the surface are accessible to lipid-hydrolysing enzyme (Maldonado-Valderrama *et al*., 2008). The chemical structure of bile salts that soluble amphiphilic molecules with an unusual molecular (Hofmann and Mysels, 1988).

Pancreatic lipase, secreted into the intestine, catalyses hydrolysis of triacylglycerols at their 1 & 3 positions, forming 1, 2-diacylglycerols and then 2-monoacyulglycerols (monoglycerides) and free fatty acids. Monoacyulglycerols and free fatty acids are absorbed by intestinal epithelial cells. Within intestinal epithelial cells triacylglycerols are resynthesized (Kimura *et al.*, 1982, Zangenberg *et al*., 2001). The activity of lipase depends on the presence of co-lipase, bile salt and calcium (Fatouros and Mullertz, 2008).

The study from Davidson *et al*. (2012) indicated that omega-3 free fatty acid formulated as emulsion has improved bioavailability during the low-fat consumption compare to omega-3-acid ethyl esters. The figure 7 shows the principle of digestion and absorption of EPA and DHA from omega-3 emulsion and omega-3 ethyl ester.

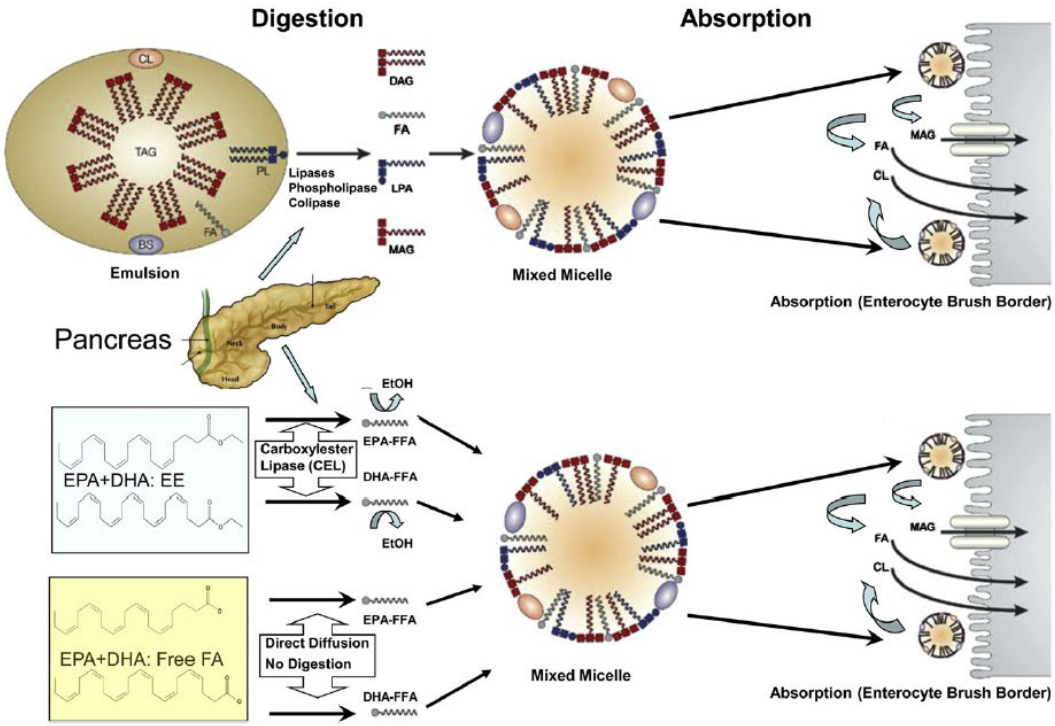

Figure 7 Digestion and absorption of EPA and DHA from omega-3 ethyl ester and omega-3 free fatty acids. (Davidson et al. 2012)

2.6 Oxidation properties

The principle of the oxidation reaction of fats and oils is because the lipids containing unsaturated acyl groups undergo oxidation reaction during the contact with oxygen to produce unpleasant odors, habits, bitterness and other undesirable tastes and some toxic and harmful compounds. First, the oil produces hydrogenated oxides under the action of oxygen in the air. The hydrogenated oxides are the main initial products of lipid oxidation, which are odorless and extremely unstable. The hydrogenated oxide then further decomposes into aldehydes, ketones, acids, and other difunctional oxides, producing an unacceptable odor that leads to rancidity of the oil. Small molecule compounds produced by oxidation of fats and oils can also be further polymerized to form dimers or multimers. For example, the oxidation product of linoleic acid is hexanal. The linoleate has a pentadiene structure, and the subunit at

the 11th position is adjacent to the two double bonds, and is sensitive to oxidation. Even at 0° or lower, linoleic acid can be oxidized (Frankel E. 2010).

Oil-in-water emulsion systems are often used to incorporate various LC3PUFA source oils in to foods with an aqueous base. However, this may create problems due to low water solubility and the chemical structure of LC3PUFA which makes them particularly susceptible to spoilage as a result of lipid oxidation (Bush, Stevenson, & Lane, 2017; Shahidi, 2015).

A great deal of recent research has focused on the suitability of LC3PUFA nanoemulsion enriched functional foods. Nanoemulsions, systems with extremely small droplet sizes have the potential to improve the bioavailability of nutrients and may have increased chemical and physical stability in comparison to systems with larger oil droplet sizes (Lane, Li, Smith, & Derbyshire, 2014; Walker, Decker, & McClements, 2015). Nanoemulsions can be created using combinations of high surfactant levels and high mechanical energy. The use of ultrasound has recently proved to be a popular high energy mechanical creation method due to low maintenance, running costs and wear and tear rates (Ghorbanzade, Jafari, Akhavan, & Hadavi, 2017; Leong, Wooster, Kentish, & Ashokkumar, 2009; Nejadmansouri, Hosseini, Niakosari, Yousefi, & Golmakani, 2016).

Nanoemulsions (size <200 nm) address the solubility problems of hydrophobic materials and provide their use in beverages and other aqueous food systems with improved bioavailability and targeted delivery (Sharif, Williams, Sharif, Khan, Majeed, Safdar, et al., 2017). However, oxidative stability is a key quality parameter for nanoemulsions containing lipid or lipophilic components that are susceptible to oxidation (Sharif, Williams, Sharif, Khan, Majeed, Safdar, *et al*., 2017, Medina, I., Satué-Gracia, M. T. and Frankel, E. N., 1999). The reduction in droplet size of the emulsion increases the surface area and enhances the interaction of the

encapsulating material with the aqueous phase, which results in an increase in the rate of oxidation. The production of secondary oxidation products (aldehydes and ketones) at the expense of oxidation of polyunsaturated fatty acids not only reduces the nutritional quality and sensory properties of the food, but may also have some impact on health (Nogueira M. S. *et al*., 2018, Kolanowski, Jaworska, & Weißbrodt, 2007, Richards A. *et al*., 2011). Oxidation not only affects the flavour of the oil, but also affects its nutritional quality and toxicity. Among the several factors that induce free radical formation and subsequent lipid auto-oxidation, light, transition metals, and temperature all have an effect. Edible oil is exposed to these factors during the extraction step used in food preparation (Gallaher J. J. *et al*., 2005).

In previous work, oil-in-water nanoemulsion systems suitable for functional food enrichment were successfully created using ultrasound with high DHA vegetarian algal oil loads up 50% (w/w) (Lane, Li, Smith, & Derbyshire, 2016). In the current study, the physical and oxidative stability of 50% (w/w) algal oil nanoemulsion systems created with ultrasound processing using natural and synthetic emulsifiers was evaluated and compared to that of bulk oil during storage at a range of different temperatures. Soy lecithin, a natural emulsifier has good emulsifying properties due to its molecular structure, which has hydrophilic and lipophilic groups and a hydrophilic-lipophilic balance (HLB) of 8 making it well suited to the successful creation of LCω3PUFA algal oil and marine based oil nanoemulsions (Coultate, 2016). Soy lecithin has been used successfully to create physically stable nanoemulsion systems in various previous studies (Ghorbanzade, Jafari, Akhavan, & Hadavi, 2017; Karthik & Anandharamakrishnan, 2016a; Lane, Li, Smith, & Derbyshire, 2016; Rasti, Erfanian, & Selamat, 2017). Tween 40 is a synthetic, non-ionic surfactant with HLB of 15.6 that has been widely used to formulate stable LCω3PUFA nanoemulsion systems (Coultate, 2016). The use of Tween 40 in the creation of LCω3PUFA nanoemulsions has also previously been

demonstrated to have a protective effect on lipid oxidation (Karthik & Anandharamakrishnan, 2016b; Lane, Zhou, Robinson, & Li, 2017; Uluata, McClements, & Decker, 2015). A review of the literature demonstrates that a study to evaluate the oxidative stability of LCω3PUFA algal oil nanoemulsions at 50% (w/w) oil loads created by ultrasound using lecithin and Tween 40 has yet to be undertaken. Therefore, the objective of the current study was to evaluate the oxidative stability of algal oil nanoemulsions created using ultrasound with lecithin and Tween 40 solely and in combination to maximise their physical and chemical stabilising properties. The research hypothesis was that the combined use of lecithin and Tween 40 would create physically stable nanoemulsion systems with small droplet ranges and enhanced oxidative stability in comparison to systems created using the emulsifiers singularly.

2.7 Current issues for emulsions to delivery nutrients

Although the delivery system of emulsion for Vitamin D and Omega-3 have been studied in previous research (Kaemi *et al*., 2007; Jacobsen *et al.,* 2010), there are current problems in the stability, oxidation and sensory palatability that need to be solved (Moore *et al.*, 1998; Lesmes *et al.,* 2010; Haham *et al*., 2012; Lane, 2013).

In addition, Lesmes *et al*., (2010) found that the lipid oxidation of emulsion also affect flavour, texture, appearance, and nutritional quality of food products, related to omega-3 enriched foods. Polyunsaturated fatty acids are generally present as oil droplets stabilised with protein or non-protein emulsifiers. For example, the lecithin is amphoteric and carries both a positive and negative charge. Tween 40 is a non-ionic, neutral surfactant and will create droplets with no charge. In the Lane's study, the lecithin had protective effect on oxidation in the omega-3 emulsion; however, the emulsion with tween 40 was less stable to

oxidation (Lane, 2013). Previous research results show that oil droplet size is an important consideration in controlling oxidation of lipids in emulsions; smaller droplets have an increased surface area and are more prone to oxidation than larger droplets (Lethuaut *et al*., 2002).

In addition, the results of sensory testing of food with omega-3 nanoemulsion by Lane (2013) showed nanoemulsion had a significantly negative effect on sensory acceptability, and the research demonstrated the sensory differences between the emulsions with different emulsifiers, for example, the nanoemulsions made with lecithin are better than Tween 40. The results from Moors et al., (1998) stated that significantly affect sensory attributes by higher oil phase of emulsion. The droplets size of emulsion is known to affect the release of lipophilic flavours from the oil droplets, where increasing the diameter correlate to an increase in aroma release (Van Ruth *et al*., 2002).

2.8 Summary

In summary, the issues of this study was focus on this three main point. Firstly, the stability of the nanoemulsion at the bioavailability for enrich nutrients. Secondly, the emulsifier and oil phase of emulsion prepared affect and control the droplet size of nanoemulsion. Furthermore, these studies found the negative effect of emulsion on the sensory attributes were also related to emulsifier and droplets size (Lane, 2013; Van Ruth *et al*., 2002). However, research in the sensory aspect of nanoemulsion were not intensively studied in the past and current studies, indicating the need for future investigations.

Referred to Lane's study (2013) the method to create a 50% oil phase nanoemulsion was developed, but the method still has some issues claimed before need to evaluate. This study was designed to evaluate the stability of two-step nanoemulsion and establish the relationship between the sensory characteristics and nanoemulsion.

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Chapter 3 The stability of an Omega-3 enriched nanoemulsion delivery system with food applications

3.1 Introduction

3.1.1 The background to the development of nanoemulsion applications

Nanoemulsions are being increasingly utilized in the food industry to encapsulate, protect and deliver lipophilic functional components, such as biologically active oils (omega-3 fatty acids) and oil-soluble vitamins (McClement & Rao, 2011, Lane *et al.*, 2012; Lane *et al.*, 2014a; Lin *et al*., 2014b). The advantages of nanoemulsions are that they are stable to droplet aggregation and resist gravitational separation, have high optical clarity and an ability to modulate product texture, bioavailability and improve the bioefficacy of the encapsulated lipophilic bioactives. Biological effects of the active ingredients can be expected to be greater when delivered in the form of a nanoemulsion instead of a normal emulsion (Acosta, 2009).

Studies (Calvo *et al*., 2004; Tomashek *et al*., 2004; Swanson *et al*., 2012) have indicated that omega-3, a set of polyunsaturated fatty acids, found in deep-sea fishes and certain plants are beneficial to human health when the consumption exceeds certain levels. Because these omega-3 fatty acids appear to have protective effects against certain chronic diseases, including arthritis pain, cardiovascular disease and the omega-3 is protective against inflammation (Senftleber *et al*., 2017; Timothy *et al*., 2013).

The two-step nanoemulsion preparetion is an oil-in-water emulsion coated with an outer layer that the additional ingredients for outer layers usually selected small molecule surfactants, proteins, polysaccharides and phospholipids as an emulsifier to coat emulsions (Klinkesorn *et al*., 2005; Aoki *et al*., 2005). The sodium alginate (SA) dissolve in the water (Smitha *et al.*,

2005); and gum Arabic (GA) which also tend to form relatively thick anionic interfaces (Guzey & McClements, 2006).

Previous studies have shown that the nanoemulsification of enriched nutrients had significant negative impacts on the sensory attributes of food (Lane, 2013), and demonstrated that the sensory characteristics may be varied with different emulsifiers and emulsion droplet size (Van Ruth et al., 2002). In addition, it was be found by Lin et al., (2016) that emulsions was destabilized at gastric phase at pH 1.6 but demonstrated a positive impact on the *in vitro* bioaccessibility of DHA from the lecithin-stabilized algal oil emulsion compare to other samples, including bulk oil (BO), a mixture of oil, water and lecithin (MIX).

The current study focuses on an investigation into the stability of nanoemulsions using selective emulsifiers and different combinations to control the droplet size, and enhance the digestibility of DHA in the algal oil nanoemulsion. Moreover, consideration should be given to improving the sensory properties of nanoemulsions by using polysaccharides as an outer layer to wrap the nanoemulsion droplet, in order to cover the negative taste and promote stability.

3.1.2 Aims

1. To develop a model of a nanoemulsion delivery system for Omega-3 using a two-step emulsion technology.

2. To establish the relationship between the sensory characteristics of a delivery system stabilized by selective emulsifiers and with designed droplet sizes.

3.1.3 Objectives

1. To evaluate the ability of selective emulsifiers to stabilize oil-in-water nanoemulsions for Omega-3 dietary oil (algal oil).

2. To evaluate the use of ultrasonic technology as a means of preparing oil-in-water nanoemulsions for Omega-3 dietary oil (algal oil).

3. To test the absorption and stabilizing properties of polysaccharides/proteins for an outer layer out of a nanoemulsion.

4. To conduct sensory testing to evaluate the sensory profile and consumer acceptability of food products incorporating of the resulting nanoemulsion, and to establish a relationship between sensory characteristics and different two-step nanoemulsion systems.

3.2 Materials and Methodology

3.2.1 Materials

Algal oil, Life DHATM S35-O300, was purchased from DSM Ltd., (Columbia, USA). Soybean liquid lecithin, NOW, (USA), Polyoxyethylenesorbitan monopalmitate (Tween 40, P1504) were purchased from Sigma-Aldrich, UK. Sodium chloride (99.5%) was purchased from ACROS, Spain. All chemicals used for the *In Vitro* digestion model were purchased from Sigma-Aldrich, UK.

3.2.2 Preparation of nanoemulsions with an outer layer coating

3.2.2.1 Oil in water nanoemulsion preparation

Nanoemulsions were prepared using omega-3 fatty acids oil following a method described by Lane (2013) with selected emulsifiers 6% (w/w), including lecithin, Tween 40, different ratios of combined lecithin and Tween 40 (90:10, 70:30 and 50:50), and Algal oil 50% (w/w) and water 44% (w/w) (Table 2). The Mixture of lecithin and algal oil (30:70) was place in a water bath at 56 ºC for 2 hours. After premixing, extra algal oil and water was added and the vessel it was replaced in the water bath for another 2 hours. During each hour, it was hand stirred for 30 seconds. The coarse emulsion was homogenised for two minutes at the maximum speed (Silverson Machine Ltd, England), and the sample was placed in a cold water cooling jacket and processed using ultrasonic processor (UP 400, Hielscher, Germany) using a 24KHz sonicator. The tip was immersed in the coarse emulsion, and then the ultrasonic processor was turned on at full power for 10 mins that smooth shaked the cooling jacket.

Table 2 Nanoemulsion ingredient ratios

Emulsion	Emulsifier (g)	Algal oil	Water (g)	Total weight (g)
Lecithin	12	100	88	200
Tween 40	12	100	88	200
LE/ TW 40 (90:10)	10.8 : 1.2	100	88	200
LE/ TW 40 (50:50)	6 : 6	100	88	200

3.2.2.2 The preparation of a nanoemulsion with outer coating layer

McClement (2012) and Ogawa et al. (2004) describe the methods for prepare a coating o layer in two steps. Firstly, the selected polysaccharides, sodium alginate and gum Arabic, which were dissolved in water at different ratios as indicated in Table 3. For all polysaccharide solution methods, powder was added into the water separately in 5 times, stirred for over 5 hours, at 60 ºC using an RT 15 power multi-stirrer (IKA WERKE GM BH & Co., Germany). Then, 10 mL polysaccharides solution was added into a 40 mL nanoemulsion, and stirred for 5 hours to make the polysaccharide coating on each nanoemulsion droplet.

Table 3 The ratio of polysaccharide solution in water and in the emulsion samples

Polysaccharide	Ratio of solution	LE/TW 9:1 Emulsion	LE/TW 5:5 Emulsion
Sodium Alginate	1%	0.2%	0.2%
Sodium Alginate	1.5%	0.3%	0.3%
Sodium Alginate	2%	0.4%	0.4%
Arabic Gum	3%	0.6%	0.6%
Arabic Gum	5%	1%	1%
Arabic Gum	7%	1.4%	1.4%
Arabic Gum	10%	2%	2%

3.2.3 *In vitro* digestion

In vitro digestion were conducted on the prepared nanoemulsion preparations with different emulsifiers. The digestion model includes the gastric phase, pH 1.6 for 60 min, and duodenal phase, pH 6.8 for 180 min. The samples tested in the digestion model were 1.0 g nanoemulsion samples (LE 100%, LE/TW), 0.5 g algal oil in bulk oil sample (BO) and MIX sample, which was a mixture of 0.5 g oil, 0.06 g lecithin and 0.44 g water as the same ration in the prepared nanoemulsion. The simulated digestion fluids (Table.4) were prepared following Nik et al.'s (2010) method, for simulated gastric fluid (SGF) containing 3.2 mg mL-1 pepsin and 12.6 mg mL-1 pyrogallol (as an antioxidant), this was adjusted to pH 1.6 with 1 M HCl then incubated at 37ºC for 60 minutes. To simulate the duodenal phase of digestion lipase from porcine pancreas, phospholipid (Soy lecithin powder) and bile extract was added to the samples to achieve a concentration of 5 mg mL-1, 3.8 mg mL-1 and 8 mg mL-1 respectively and the pH adjusted to 6.8 with NaOH.

Table 4 Composition of the simulated gastric (SGF), duodenal (SDF), and bile (SBF) fluids

Component	**SGF (mM)**	**SDF (mM)**	**SBF (mM)**	**Final mixture Concentration (mM)**
NaCl	94.2	240.0	180.0	130.5
NaH_2PO_4	4.4	-	-	1.4
$NaHCO_3$	-	80.0	137.0	45.7
KCl	22.1	15.1	10	13.0
KH_2PO_4	-	1.2	-	0.4
$CaCl_2.2H_2O$	5.4	2.7	3.0	3.0
NH_4Cl	11.4	-	-	3.5
$MgCl_2$	-	0.5	-	0.2
Total	137.6	340.0	330.0	197.5
pH	1.3	8.1	8.2	6.5

The digestion tests were performed in bottles with closed lids were placed in a shaking water bath (Grant Instrument Ltd, Cambridge, England) and maintained at 37ºC and a shaking speed of 60 rpm. LE50, BO and MIX were made up into 5 mL meal samples with water containing 10wt % oil, and these were warmed at 37ºC for 15 minutes before adding 7.5 mL SGF and then being placed in a shaking water bath. After 1 hour, 7.5 mL SBF and 3.5 mL SDF were added for the duodenal digestion and pH as adjusted to 6.8 then kept in water shaking bath at 37ºC for 3 hours.

3.2.4 Separation of aqueous phase bile salt micelles

Droplet size measurement was conducted during the duodenal phase digestion. Droplet size testing used an ultracentrifuge to separate the water phase and undigested the duodenal digestion fluid. The digested sample was centrifuged at 144, 000g at 7 ºC for 1h using a Ultracentrifuge SW 32Ti Rotor (Beckman Coulter, USA) to separate indigestion oil from the aqueous phase, water phase and pellet (Lin et al, 2014). Samples were collected individually from the upper oil and lower water phases using separate pipettes and transferred to different labelled bottles. All the samples were then saved until the particle size and fatty acid composition measurements could be made.

3.2.5 Measurement of particle size

Droplet size was report as $D_{3,2}$ and $D_{4,3}$. $D_{3,2}$ is the volume/surface diameter mean or Sauter mean. It provides a measure of mean diameter specific surface area. $D_{4,3}$ is the mean diameter over volume or DeBroukere mean. It provides a measure of droplet specific surface area, which defined in following equation.

$$d_{32} = \frac{\sum_i d_i^3}{\sum_i n_i d_i^2}, \quad d_{43} = \frac{\sum_i n_i d_i^4}{\sum_i n_i d_i^3}$$

ni is the number of droplet and di is the diameter. The droplet size of emulsion samples before, during and after digestion were determined using a Mastersizer MS2000 laser light-scattering analyser (Malvern Instrument Ltd, UK) with a small sample dispersion unit set around 1650 to 2000 rpm (Akhtar et al., 2006; Lane, 2013). For the emulsion samples, an absorption parameter value of 0.001 using a refractive index ratio of 1.488 for algal oil.

3.2.6 Scanning Electron Microscopy (SEM) of nanoemulsion samples

The emulsion samples were diluted 100 times in distilled water, one drop of each diluted sample was placed onto individual glass slides, which were placed onto a table glass desiccator for over 12 hours at room temperature to dry the emulsion samples. The samples were scanned using Scanning Electron Microcopy (a Carl Zeiss Supra 40 VP SEM) using a method developed by Mathapa & Paunov (2013) and the data was collected using Carl Zeiss Smart SEM, V05.01.08 software.

3.2.7 Statistical analysis

All measurements were performed in triplicate. Results are expressed as mean ± standard deviation. The analysis of Post-Hoc test (ANOVA, SPSS statistic 21) was used to compare sample means to check for significant differences in distribution of testing data.

3.3 Results

3.3.1 Effect of different emulsifiers on the particle size in nanoemulsions.

The droplets size average diameter of nanoemulsions with different emulsifiers and variable emulsifier combinations are shows in Table 5. $D_{3,2}$ and $D_{4,3}$ values, which were explained in the method section, were significantly affected by emulsifiers ($P < 0.05$). The results in Table 5 and Fig. 8 show the higher ratio Tween 40 of combine emulsifier with lecithin could control the nanoemulsion droplet size. However, the mean droplet size of nanoemulsion that only contain Tween 40 was significantly higher than the nanoemulsion contain the combined 3% w/w lecithin and 3% w/w Tween40, which the value of $D_{3,2}$ were 0.187 μm and 0.173 μm.

Table 5 The average droplet size of nanoemulsions with different emulsifiers

	LE 100	LE/TW 90:10	LE/TW 70:30	LE/TW 50:50	TW 100
Volume median diameter (D0.5) (μm)	0.853	0.646	0.438	0.222	0.275
Diameter volume accumulation 90% (D0.9)	2.057	1.765	1.063	0.792	1.232
% volume droplet > 1 micron	42.619	31.421	12.037	4.358	16.182
Surface weighted mean D[3,2] (μm)	0.266	0.234	0.235	0.173	0.187
Volume weighted mean D[4,3] (μm)	0.953	0.784	0.513	0.351	0.517

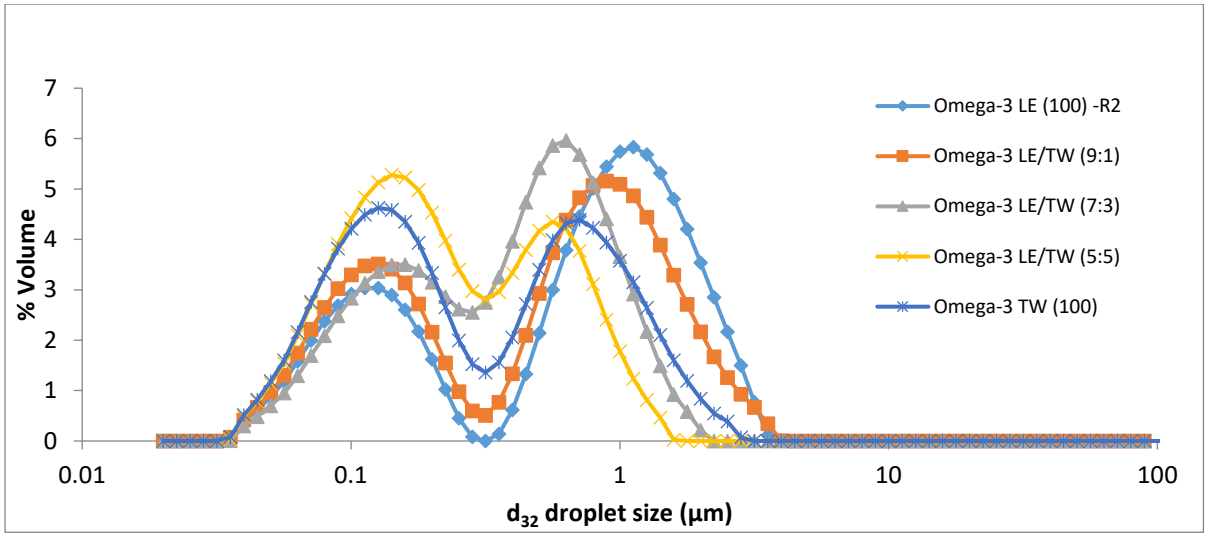

Figure 8 The particle size distribution of nanoemulsions with different emulsifiers and different combinations of emulsifiers.

3.3.2 SEM of Nanoemulsions

Figure 9 and figure 10 show the droplet of LE/TW 9:1 nanoemulsion with gum Arabic and sodium alginate. Image A shows dark particles that represent the nanoemulsion droplet. The white tiny particles between and surrounding the dark particles in images B, C and D indicates the droplets of nanoemulsion with polysaccharide coating.

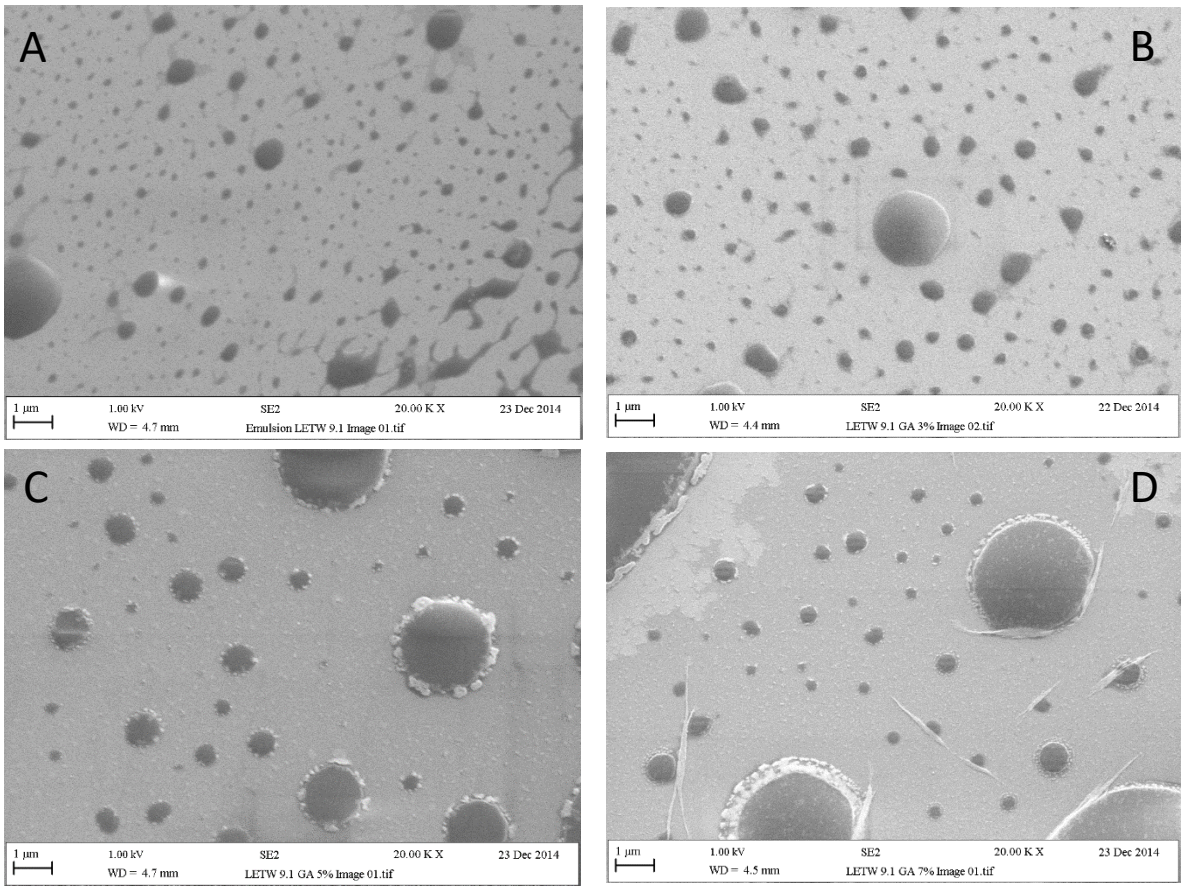

Figure 9 SEM of nanoemulsion with and without coating Gum Arabic (Omega-3 nanoemulsion LETW 9:1 (A), nanoemulsion coating 3% Gum Arabic solution; (B), nanoemulsion coating 5% Gum Arabic solution; (C), nanoemulsion coating 7% Gum Arabic solution (D).

Similar white particles present in Figure 9 were also present in images B and C of Figure 10. However, the Fig. 10 image D shows that sodium alginate was too concentrated (2%) to coat the nanoemulsion droplets.

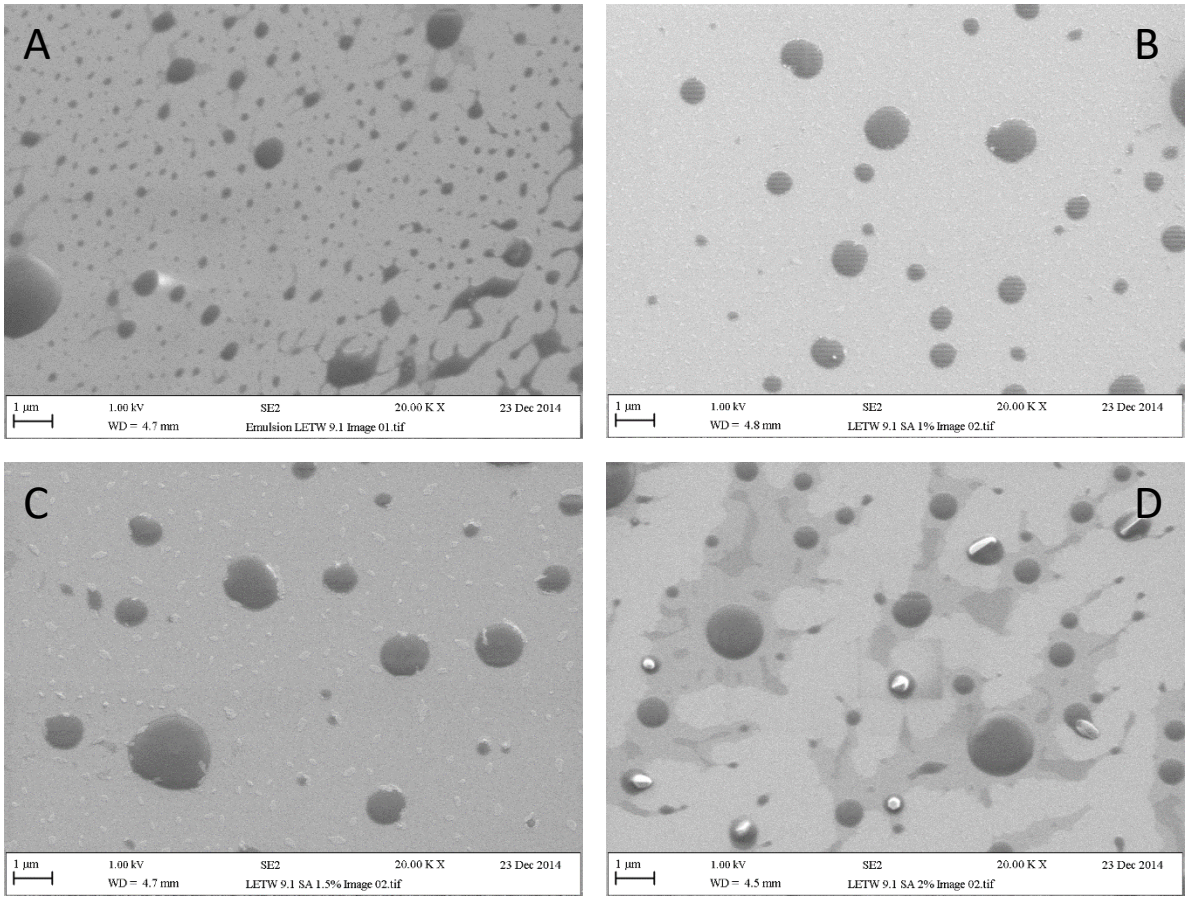

Figure 10 SEM of nanoemulsion with alginates. Nanoemulsion coating 1% sodium alginate solution (B), nanoemulsion coating 1.5% sodium alginate solution (C), nanoemulsion coating 2% sodium alginate solution (D)

3.3.3 Digestive Stability and diameter droplet sizes of nanoemulsion with different emulsifiers during *in vitro* digestion

Digestive stability, in terms of droplet sizes of nanoemulsion with different emulsifiers was investigated during the *in vitro* digestion experiments. The results show that the emulsifiers dramatically affected the stability of nanoemulsions during digestion as shown in Fig. 11. At a gastric pH of 1.6, the nanoemulsion with 100% LE destabilized during 5 min to 60 min exposure to SGF (Fig. 11B, C). There was a clear oil phase separated on above the aqueous phase (Fig. 11C). The other vials, which are the duodenal phase at 5 min (Fig. 11 D) and 60 min (Fig. 11E), show significant separation into two phases.

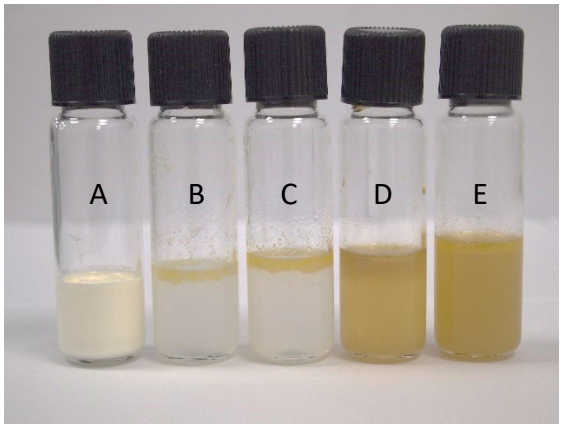

Figure 11 The appearance of Omega-3 nanoemulsion (100% LE) during digestion with pH 1.6 gastric phase (Omega-3 nanoemulsion (100% LE) before SGF addition (A), after 5 minutes of pH 1.6 gastric phase (B), after 60 minutes of pH 1.6 gastric phase (C), after 5 minutes of pH 6.8 duodenal phase (D), and after 60 minutes of pH 6.8 duodenal phase (E).)

For comparison, the stability of omega-3 nanoemulsion with LE/TW 5:5 was also tested in digestion experimental model, under the same conditions, the results show no visible separation during the digestion, show in Fig 12.

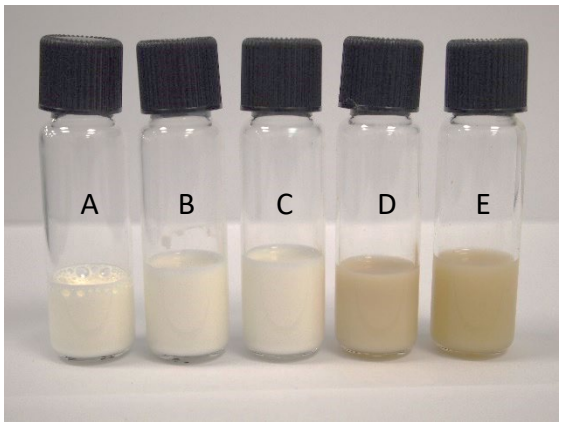

Figure 12 The appearance of Omega-3 nanoemulsion (100% LE) during digestion with pH 1.6 gastric phase. (Omega-3 nanoemulsion (LE/TW 5:5) before SGF addition (A), after 5 minutes of pH 1.6 gastric phase (B), after 60 minutes of pH 1.6 gastric phase (C), after 5 minutes of pH 6.8 duodenal phase (D), and after 60 minutes of pH 6.8 duodenal phase (E).)

The droplet size measurements were conducted from before digestion to finish the duodenal phase (Table 5, 7and Fig. 13, 14, 15, 16). The measurements of droplet size with two nanoemulsions respectively stabilized by LE/TW (9:1) and LE during the digestion are

shown in the Table 6 and Table 7. At the beginning (5 min) and end (60 min) of the gastric phase (GP), the droplet size (D3,2) of the nanoemulsion with 100% lecithin increased from 0.340 µm (before digestion) to 19.4 µm (GP 5 min) and 21.0 µm (GP 60 min). The mean droplet diameters before digestion and after 5 min gastric digestion were significantly different ($P < 0.05$). Nevertheless, the droplet size of nanoemulsions prepared with combined emulsifiers (LE/TW 5:5) were stabilized around 0.202 ± 0.015 µm throughout digestion under the same conditions.

Table 6 Mean of diameters (D3,2) of LE/TW 9:1 emulsion, LE 100% emulsion during digestion.

Algal oil	LE/TW 9:1 Emulsion	LE 100% Emulsion
Digestion stage	**$D_{3,2}$(µm)**	**$D_{3,2}$(µm)**
Before digestion	0.289	0.340
Gastric 5 min	0.274	19.4
Gastric 30 min	0.272	35.7
Gastric 60 min	0.306	21.2
Duodenal 0 min	0.344	0.459
Duodenal 30 min	0.405	11.5
Duodenal 60 min	0.257	12.8
Duodenal 120 min	0.206	11.9
Duodenal 180 min	0.221	17.2

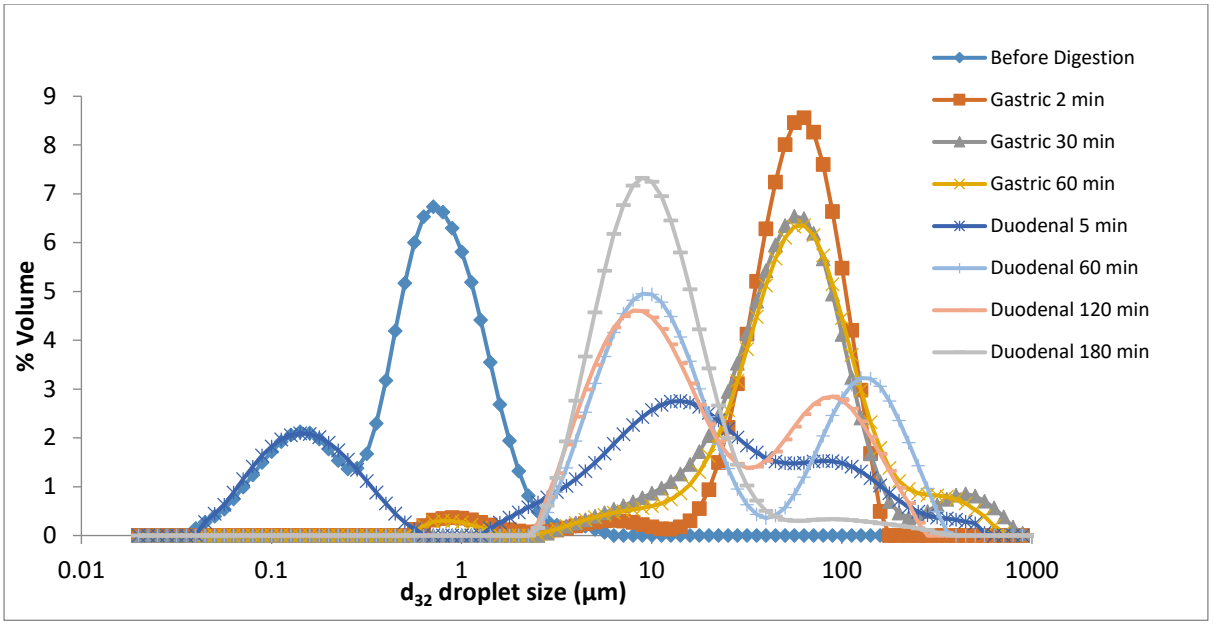

Figure 13 Drop size distribution of omega-3 LE 100% nanoemulsion during digestion

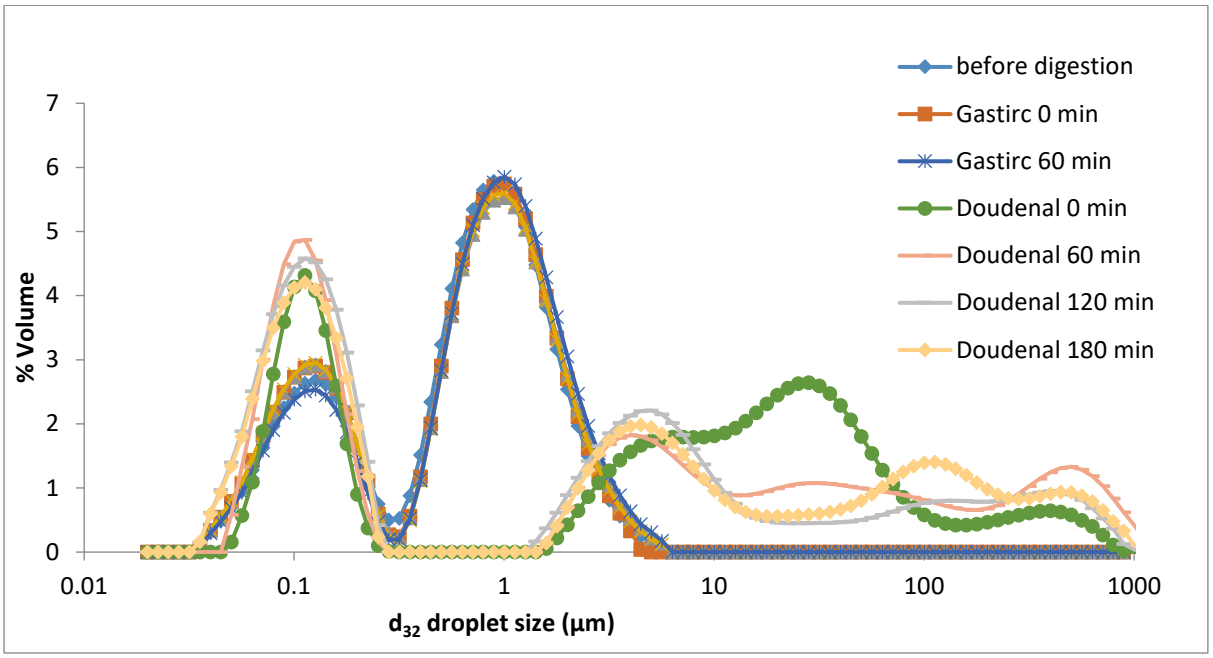

Figure 14 Droplet size distribution of omega-3 LE/TW 9:1 nanoemulsion during digestion

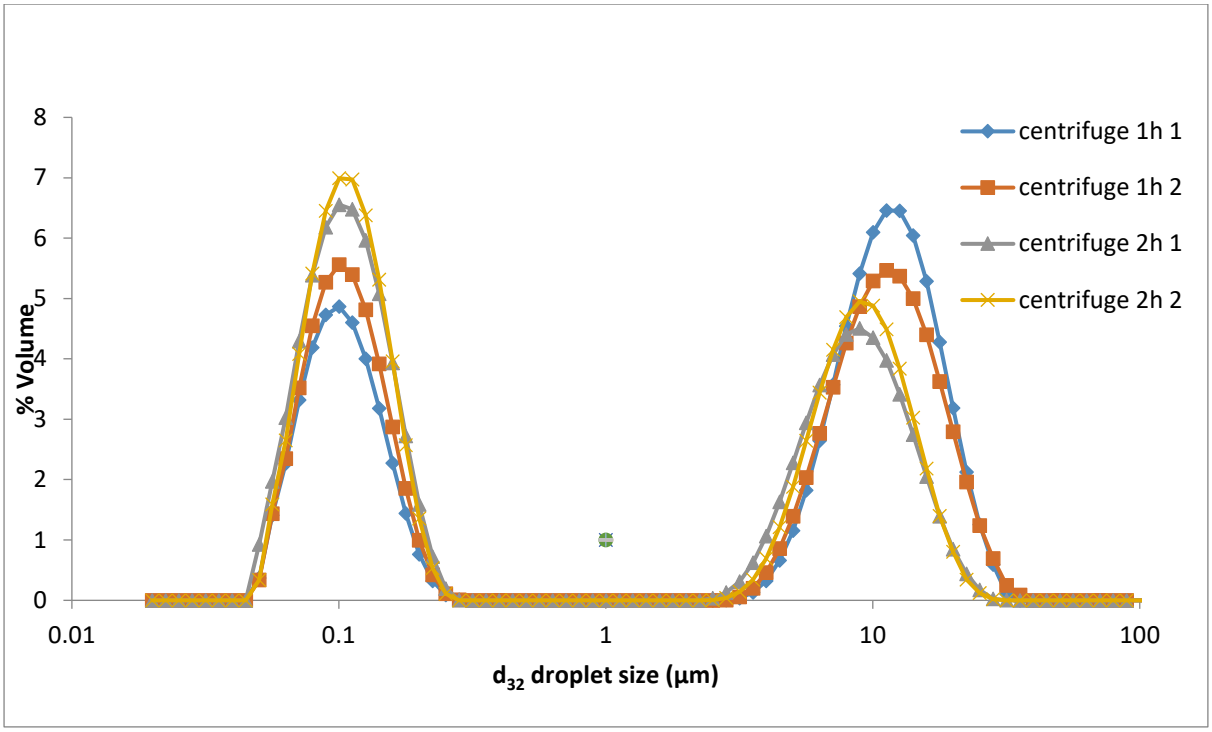

Figure 15 The particle size of aqueous phase with 60 min and 120 min duodenal digestion after centrifugation.

Interestingly, the droplet size of nanoemulsion (100%) at the beginning of duodenal digestion phase (0 min) decreased to ($D_{3,2}$) 0.459 µm.

Figure 14 shows that the particle size of nanoemulsion (LE/TW 9:1) has a major peak around 0.112 µm at duodenal digestion, and also have other peaks after 1.588 µm. The droplet size distribution in the aqueous phase after the nanoemulsion was digested and centrifuged, is shown in the Figure 15, has two peaks observed around 0.100 µm and 11.2 µm.

The results in Table 7 show that the droplet size in the nanoemulsion with LE/TW 5:5, bulk oil and MIX during digestion showed two phases. Size increases during the gastric digestion phase and the falls immediately upon the change to duodenal digestion and the begins to rise again. The droplet size in this LE/TW 5:5 emulsion appears to be stable throughout the gastric phase of the digestion experiment (Table 7 and Figure 16). During the duodenal

phase, the same sample showed a small decline in the droplet size with time (Table 7). The nanoemulsion sample had a part of droplet are concentrated at 0.3 µm, compare to bulk oil and MIX sample, emulsion sample was more stable during the gastric phase and duodenal phase.

Table 7 Mean of diameters (D3,2 and D4,3) of LE/ TW 5:5 nanoemulsion, bulk oil and MIX during digestion.

LE/TW 5:5 Algal oil	nanoemulsion	Bulk oil	MIX
Digestion stage	**$D_{3,2}$ (µm)**	**$D_{3,2}$(µm)**	**$D_{3,2}$(µm)**
Before digestion	0.193	105.5	30.6
Gastric 0 min	0.226	104.4	40.9
Gastric 20 min	0.204	94.4	17.3
Gastric 40 min	0.198	152.3	16.7
Gastric 60 min	0.189	89.9	21.0
Duodenal 0 min	0.361	16.3	26.1
Duodenal 30 min	0.323	33.2	25.1
Duodenal 60 min	0.301	35.5	18.0
Duodenal 120 min	0.192	26.8	19.1

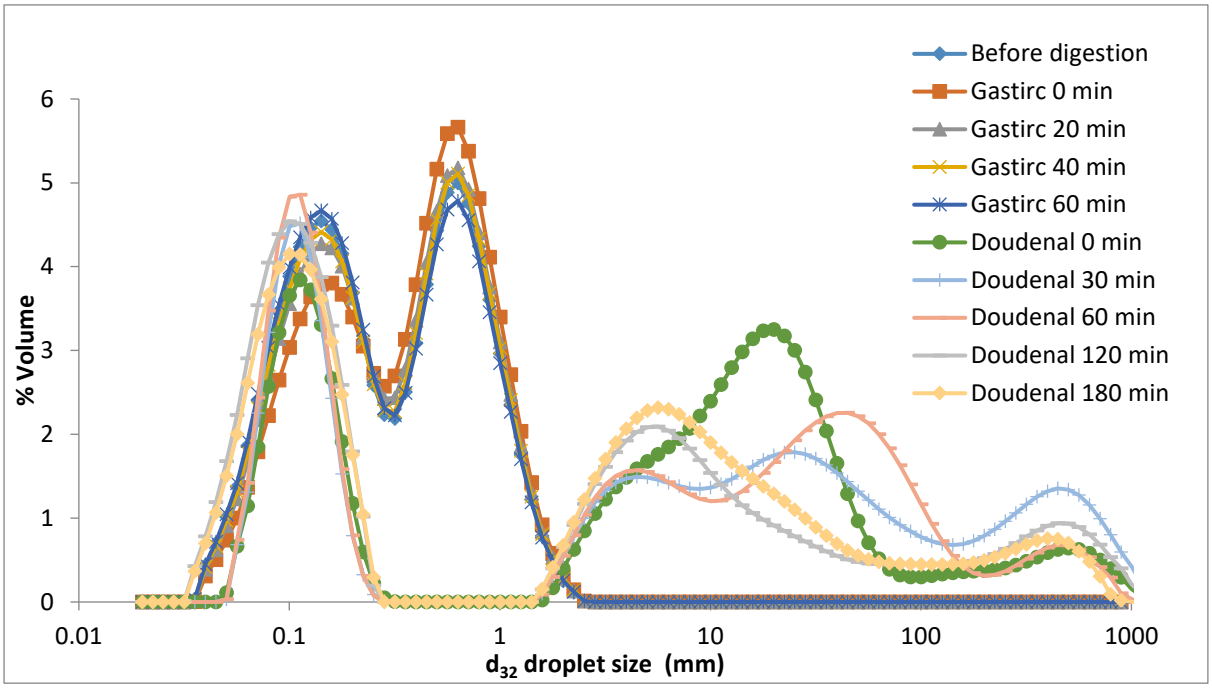

Figure 16 Droplet size distribution of omega-3 LE/TW 5:5 nanoemulsion during digestion

3.4 Discussion

The results show that the nanoemulsion stabilized by 100% lecithin (100% LE) significantly phase-separated during the gastric phase of the digestion experiment (pH 1.6) (Fig. 11), which is in agreement with the report of Lin *et al*. (2014). In these results, the nanoemulsion (100% LE) was destabilized and the oil was released to forming a separate upper phase. Mantovani et al. (2013) claimed that the soy lecithin contains an anionic phosphate group. At pH 5, the phosphate group has a negative charge and the phosphate ester has a positive charge (charge on N) (Fig. 17), this enables the emulsions to be stable. When the pH of the emulsion is under 3, the negative charges of the phosphate group of lecithin are neutralized allowing the positively charged neo-pentane to provide electrostatic repulsion. Therefore, nanoemulsions stabilized by lecithin are not stable below pH 3. Thus at pH1.6 the phases separate (Fig. 17).

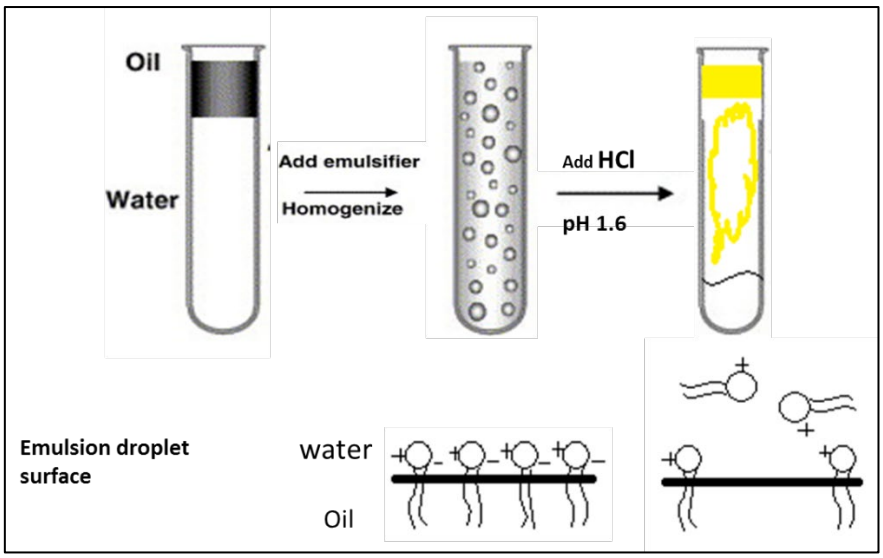

Figure 17 The structure of the nanoemulsion stabilized by lecithin at pH of 1.6

The omega-3 nanoemulsion (LE/TW 5:5) was more stabilized, which shows from the results. The Tween 40 is a polyoxyethylene (20) sorbitan monopalmitate, which is a water soluble

emulsifier (HLB 15.6). A previous study (Whitehurst, 2004) showed that the chemistry of polysorbates and sorbitan ester are closely related and perfectly complement each other thus the combination of the two emulsifiers works best for stabilizing emulsions. In addition, the two emulsifiers can pack the interface and mechanical strength more densely than alone. Tween 40 is a nonionic polysorbate emulsifier, which has no effect on electrostatic stabilization. As Fig. 18 shows nanoemulsions coated with LE/TW 5:5 at pH 1.6 can be stable, because of each positively charged lecithin has a Tween 40 molecule has an adjacent lecithin molecule which naturalizes the ionic repulsion effect produced by the positive charges.

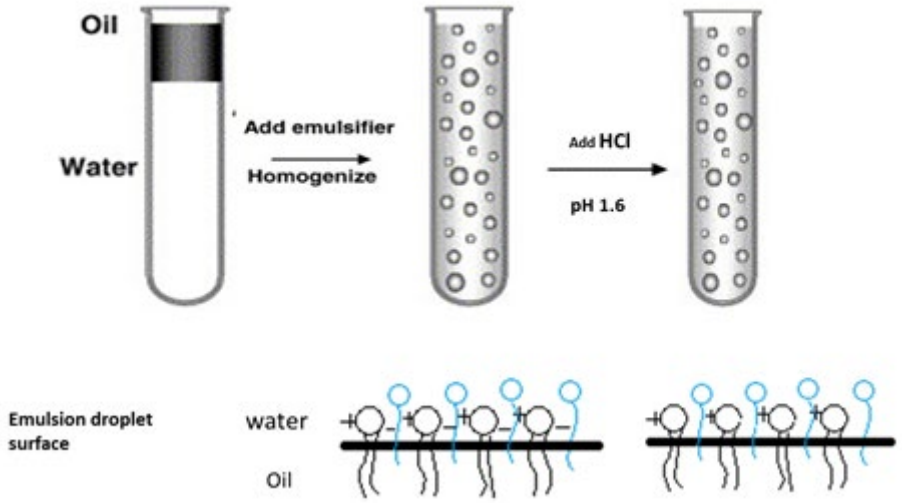

Figure 18 The structure of the nanoemulsion stabilized by 50% lecithin and 50% Tween 40 at pH 1.6.

The size difference between omega-3 nanoemulsion stabilised by 6% lecithin (LE), 6% Tween 40 (TN) and 3% lecithin combined with 3% Tween 40 (LE/TW) agrees with the observation that the lecithin is less effective at reducing droplet size than lecithin combined with Tween 4. The molecular weight of lecithin is bigger than Tween 40, which the nanoemulsion droplet using lecithin will form a thick surface.

Furthermore, Tween 40 is able to affect the droplets size, because the higher HLB value of the emulsifier combined with another emulsifier would create a natural balance giving a system with much a smaller droplet size. The results of the droplet size measurements show that the droplet size of nanoemulsions with different ratios of the combinations of emulsifiers (LE and LE/TW) dramatically changed, which means that the droplet size can be controlled by the combination of emulsifiers.

When the oil-in-water emulsion using emulsifier is positively or negatively charged, the surface of the emulsion droplets will have charge on the surface and able to mix with an oppositely charged polyelectrolyte solution to produce a second layer.

In this study, the polysaccharides selected to form the outer layer were sodium alginate, a negatively charged, water soluble polysaccharide (Smitha *et al,* 2005) and gum Arabic (GA), sodium alginate is a water soluble polysaccharide contain negatively charge (COO-) when the sodium alginate (SA) dissolve in the water (Smitha *et al.*, 2005). The SEM scans show that the different ratios of coating solutions, which were 1%. 1.5% and 2% sodium alginate and 3%, 5% and 7% gum Arabic, have significant effects on the nanoemulsions which the dark droplets had significant white spot cover on, compare to the dark droplet without any white spot show the nanoemulsion without coating (Fig. 9 and Fig. 10). Comparing all the images, the bright droplets in the specific images (Fig. 9 C, D and Fig. 10 B, C) are well coated with the polysaccharides. Nevertheless, there were still some larger droplets that were not fully covered by polysaccharides. The current research also examined the charge on the surface of droplets with increasing concentrations of polysaccharides, and observed the coverage of droplets improved as the concentration of the polysaccharides increase. The best coverage was observed at 7% GA and 2% SA. (Fig. 9D and Fig. 10D).

3.5 Conclusion

This study in this chapter examined the stability of nanoemulsion with different emulsifiers and their combinations using an *in vitro* digestion approach. First of all, it was observed that the nanoemulsion has been stabilised with selected emulsifiers (LE/TW 9:1 LE/TW 7:3 & LE/TW 5:5,) and the drop size of nanoemulsion decreased with an increase in the TW ratio of combined LE-TW. Secondly, the omega-3 nanoemulsion developed with the LE/TW combination at ratio of 5:5 was found to be stable during the *in vitro* digestion of gastric phase at pH 1.6, for the whole duration of 60 min. However, the stability of nanoemulsions was not maintained in the duodenal phase at pH 6. Thirdly, during the coating formation, it was demonstrated that both the sodium alginate and gum Arabic were able to fully coat the nanoemulsion small droplets.

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Chapter 4 Factors which improve the digestibility of Omega-3 enriched emulsions studied using an *In vitro* digestion model

4.1 Introduction

4.1.1 Background

A number of studies (Calvo *et al*., 2004; Tomashek *et al.*, 2004; Swanson *et al.*, 2012; Lane *et al*., 2014a; Lane *et al*., 2014b) reported that long chain omega-3 polyunsaturated fatty acids (LC3PUFA) are beneficial to human health when consumption reaches certain levels. Benefits include reducing the coronary heart disease (CHD), cardiovascular disease (CVD), chronic and inflammatory disease risks. DHA is one of the most important omega-3 fatty acids due to its role in brain development and in the functioning of the retina (Karthik and Anandharamakrishnan, 2016). Omega-3 can protect against cardiovascular disease, lowering blood pressure and heart rate, lowering blood triglycerides reducing inflammation (Defilippis and Sperling, 2006; Lee *et al.,* 2008). The previous studies found that Omega-3 fatty acid consumption is decreasing whilst there has been a substantial increase in the consumption of Omega-6 fatty acids (Sinn *et al*., 2011). Nanoemulsions are being increasingly utilized in the food industry to encapsulate, protect and deliver lipophilic function components, such as biologically-active lipids (omega-3 fatty acids) (McClement & Rao, 2011). Some current studies suggested that the daily intake of 250mg DHA and EPA from food has beneficial effects on maintenance cardiac function (Welch *et al.*, 2007, EFSA Journal DHA/EPA related health claims, 2010). For the function of Omega-3 fatty acid to maintenance the normal blood pressure and fasting on concentration of blood triglycerides, the daily intake of DHA combined EPA need achieve 2000 mg per day (Defilippis and sperling, 2006, EFSA Journal DHA/EPA related health claims, 2011).

An initial study (Lane *et al*., 2014) found that long chain omega-3 polyunsaturated fatty acids absorption from the nanoemulsion was significantly higher from than from the bulk oil, based on a single-blind, randomised crossover trial, demonstrating that nanoemulsion was a powerful way to increase long chain omega-3 polyunsaturated fatty acids absorption. However, the results of *In vitro* digestion studies with omega-3 enriched nanoemulsions showed that the nanoemulsion was destabilised at low pH (pH= 1.6) at the gastric phase, which resulted in a reduction of DHA hydrolysis compared with the stable nanoemulsion at gastric phase at pH 4 (Lin *et al*., 2014). This study indicates that the stability of the nanoemulsion plays a key role in the hydrolysis of DHA for the further absorption. Therefore, the aim of current study focused on further improvement of the stability of nanoemulsion using selected emulsifiers and combinations of emulsifier to control the droplet size during digestion.

4.1.2 Objectives

To improve the stability of nanoemulsions containing omega-3 when exposed to a low pH environment (pH 1.6)

To improve the digestibility of (DHA) from the omega-3 enriched nanoemulsions during the *In vitro* digestion experimental module.

4.2 Materials and methods

4.2.1 Materials

Algal oil, (Life DHATM S35-O300) was purchased from DSM Ltd., (Columbia, USA). L-α-Phosphatidylcholine (P3644-100G) of soybean, Type IV-S. ≥30% (enzymatic), Polyoxyethylenesorbitan monopalmitate (Tween 40, P1504) were purchased from Sigma-Aldrich, UK. Sodium chloride (99.5%) was purchased from ACROS, Spain. Hexane (HPLC Grade) was purchased from Fisher Scientific, (UK). Methanol (HPLC Grade), Sulphuric acid 95%, Sodium sulphate anhydrous were purchased from VER BDH PROLABO chemicals, EC (UK). Sodium chloride (99.5%) was purchased from ACROS (Spain).

4.2.2 Preparation of nanoemulsion

The nanoemulsion (LTN) of the omega-3 fatty acids oil was prepared using Lane's (2013) method with selected 6% (w/w) of emulsifiers including combined equal ratio of Tween 40 and lecithin, 50% (w/w) of Algal oil (Life DHA^{TM} S35-O300, DSM Ltd., Columbia) and 44% (w/w) of water. The combination of emulsifiers and algal oil in 30:70 ratios were place in a water bath at 56 ºC for 2 hours. After premixing, extra algal oil and water was added in and it was placed in the water bath for another 2 hours. During each hour, it was hand stirred for 30 seconds. The coarse emulsion was homogenized for two minutes at a maximum speed (1200 rpm) in a homogenizer (Silverson Machine Ltd, England), and the sample was processed in an ultrasonic processor (BSP-1200 Ultrasonic processor, Industrial Sonomechnics Ltd , NY, USA) at 19650Hz for 10 minutes (Figure 19).

Figure 19 BSP-1200 Bench-scale Ultrasonic liquid processor.

4.2.3 *In vitro* digestion

The samples were 1g of the LTN nanoemulsion and 1g of a compositionally equivalent MIX of 0.5g algal oil, 0.03g Lecithin, 0.03g Tween 40 and 0.44g water without processing. Simulated digestion fluids were prepared as described in table 2. LTN and MIX were made up to 5ml meal sample with water to contain 10%wt oil, and were kept at 37 $^{\circ}$C in water bath for 15min, then 7.5 ml simulated gastric fluids (SGF) with dispersed pepsin, and pepsin was added to achieved a digestion mixture containing 3.2 mg/ml pepsin and 12.6 mg/ml pyrogallol (as an antioxidant). The pH of the digestion mixture was lowered to 1.6, which represents the typical state of an empty stomach and the gastric digestion of the mixture started in shacking water bath at 37 $^{\circ}$C and 200 rpm. Following the gastric digestion, 3.5ml of simulated bile fluid (SBF) with phospholipids and 7.5ml simulated duodenal fluid (SDF) with pancreatin and pancreatic lipase were added to digestion mixture to achieve 10 mg/ml bile extract, 3.8mg/ml phospholipids 5 mg/ml pancreatin and 6 mg/ml pancreatic lipase. The pH

of this mixture was adjusted to 6.85 with the addition of 1 N NaOH and the digestion mixture of the duodenal phase was kept at 37 °C for 3 h. After digestion, all the digested samples were kept in 4 °C refrigerator before centrifuging.

Table 8 Composition and pH of the simulated gastric (SGF), duodenal (SDF), and bile (SBF) fluids used to minic fed state condition and concentration of constituents in the digestion mixture after emulsion addition.

Component	SGF (mM)	SDF (mM)	SBF (mM)	Final mixture Concentration (mM)
NaCl	94.2	240.0	180.0	130.5
NaH_2PO_4	4.4	-	-	1.4
$NaHCO_3$	-	80.0	137.0	45.7
KCl	22.1	15.1	10	13.0
KH_2PO_4	-	1.2	-	0.4
$CaCl_2.2H_2O$	5.4	2.7	3.0	3.0
NH_4Cl	11.4	-	-	3.5
$MgCl_2$	-	0.5	-	0.2
Total	137.6	340.0	330.0	197.5
pH	1.3	8.1	8.2	6.5

4.2.4 Measurement of droplet size

The droplet size of nanoemulsion samples before, during and after digestion were determined by the Mastersizer 3000 laser light-scattering analyzer (Malvern Instrument Ltd, UK) with a small sample dispersion unit at 2400 rpm (Lane, 2013; Akhtar et al., 2006). For the emulsion samples, an absorption parameter value of 0.001 using a refractive index ratio of 1.488 for algal oil. Droplet size was reported as $D_{3,2}$ and $D_{4,3}$. $D_{3,2}$ is the volume/surface diameter mean or Sauter mean. It provides a measure of mean diameter specific surface area. $D_{4,3}$ is

the mean diameter over volume or DeBroukere mean. It provides a measure of droplet specific surface area.

4.2.5 Isolation of aqueous phase from the digested fluid

Samples (23.5ml per sample) were transferred into the centrifuge tubes and balanced. The samples were centrifuged at 144, 000g at 7 $^{\circ}$C for 1 h using an Ultracentrifuge SW 32Ti Rotor (Beckman Coulter, USA) to separate the undigested oil from the aqueous phase. After centrifugation, the upper oil phase was collected using a pipette carefully. The volume and weight of aqueous phase were measured with serological pipette and all the samples were stored in closed containers under -20 ^{0}C for the further analysis.

4.2.6 Extraction of lipid of aqueous phase

Lipid extraction were performed based on the Lin et al. (2014) method with slight modifications as described. Firstly, 3 ml Hexane-methanol (1:2) was added to 1 ml of the aqueous phase. The mixture was vortexed for 60s. 1 ml Hexane was added, and samples were vortex for 15s. Phase separation was facilitated by adding 1 ml 0.5% NaCl followed by 30s of vortexing. Finally, samples were centrifuged at 2000g for 6 min, and aliquots from the hexane phase were withdrawn and kept at -20 $^{\circ}$C until analysis.

4.2.7 Determination of fatty acid composition by GC

Lipid extraction was performed using the derivatisation of fatty acid for FAME analysis method (2015) which was developed at the NoWFood research centre with slight modifications. 1.0 g sample of Hexane extract was added to 10 ml Reagent A (2.5% w/v KOH solution in Methanol). Tightened the top and the tubes placed into Kevlar sleeves into Mars 6 microwave (CEM Ltd., UK). The temperature was increased to 90 $^{\circ}$C over 5 min and held for 10 min. After cooling to room temperature, 15ml reagent B (2% sulphuric acid v/v in

Methanol) was added. The sample tubes were resealed and placed in the microwave. The temperature was increased to 120 °C and held for 6 min. After cooling to room temperature, 10ml Hexane was added and the inverted once. Sufficient saturated salt solution was added to bring the hexane layer to the top of the tube. The upper hexane layer containing the fatty acid methyl esters was separated for GC analysis.

4.2.8 GC-FID analysis

The samples were analysed using a GC Clarus 480 system (PerkinElmer Inc, USA) equipped with an auto sampler, Flame Ionization Detector (FID) and a 30 m, 0.25mm id 0.25 µm film thickness GC capillary column (SGE Analytical Science Pty Ltd, Australia) and Total Chrom Navigator software system (Version 6.3.2 PerkinElmer Inc, USA). The injector and detector temperatures were 220 ºC and 250 ºC respectively with 1.5 µl injection for each single time and the hydrogen flow pressure was set at 8.4 psi. The column temperature was programmed to increase from 60 to 170 ºC at a rate of 20 ºC/min and to 200 ºC at a rate 1 ºC/min, held at 200 °C for 1 min; the total run time was 36.5 min. The fatty acids were identified by reference to the retention time of standards (Supelco 37 Component FAME Mix, Sigma, UK). Analysis was performed in triplicate on individual vials for each time point.

4.2.9 Statistical analysis

All measurements were completed in triplicate. Results are expressed as mean ± standard deviation. The analysis of Post-Hoc test (ANOVA, SPSS statistic v 24) was used to compare sample means to check for significant differences in the distribution of data, and compare the all data from droplet size testing of nanoemulsion.

4.3 Results

4.3.1 *In vitro* digestibility of different samples

One part of this study aimed to create a nanoemulsion LTN with combined emulsifiers (3% lecithin and 3% Tween 40) in order to improve its stability during the Gastric stage at the lowest pH. The particle size measurements were carried out on samples throughout the digestion process for pre-treatment to the post duodenal digestion stage (Table 9, Fig. 20, 21, 22, and 23).

Table 9 Mean droplet diameter (D3,2 and D4,3) of LTN and MIX during digestion with gastric pH 1.6.

Samples	**LTN**		**MIX**	
Digestion stage	**$D_{3,2}$ (µm)**	**$D_{4,3}$(µm)**	**$D_{3,2}$(µm)**	**$D_{4,3}$(µm)**
Before digestion	0.267±0	0.300±0	73.6±6.98	84.73±8.24
Gastric 5 min	0.245±0.001	0.279±0.001	50.1±1.81	64.37±2.65
Gastric 60 min	0.238±0.01	0.272±0.01	28.83±0.06	36.50±0.10
Duodenal 5 min	0.326±0.006	0.383±0.008	27.30±0.30	34.57±0.40
Duodenal 60 min	14.20±0.26	20.90±0.17	21.67±0.57	30.27±0.76
Duodenal 120 min	10.31±0.52	18.37±0.67	19.82±0.10	27.43±0.15
Duodenal 180 min	8.87±2.56	20.07±3.48	18.97±8.19	32.53±14.28

Table 9 and figure 22, 23 shown the droplet size $D_{3,2}$ and $D_{4,3}$ distributions for LTN and MIX during digestion in the *In vitro* model. During the gastric stage from 5 min to 60 min, the droplet size $D_{3,2}$ and $D_{4,3}$ of LTN is significantly different ($p < 0.05$). The droplet size of the LTN and MIX samples had decreased from before digestion to after gastric phase 60 min. The average diameter of the LTN digested during duodenal phase is shown in Table 9, where

$D_{3,2}$ and $D_{4,3}$ were significantly affective by digestion time ($p < 0.05$). Which means the emulsion system of LTN was destroyed at beginning of the duodenal phase.

The droplet size distribution of the MIX sample during the *In vitro* digestion show in table 2 and figure 6. At the beginning (5 min) and end (60 min) of the gastric phase (SG), the droplet size $D_{3,2}$ of MIX are decrease from 73.6±6.98 µm (before digestion) to 59.1±1.81µm at SG 5 min and further to 28.83±0.06 µm at SG 60 min. The mean droplet diameters before digestion and 5 min gastric digestion were significantly different ($P < 0.05$). In contrast, the droplet size of nano-emulsion with combined emulsifiers (LTN) remain stable around 0.238 ± 0.01 µm.

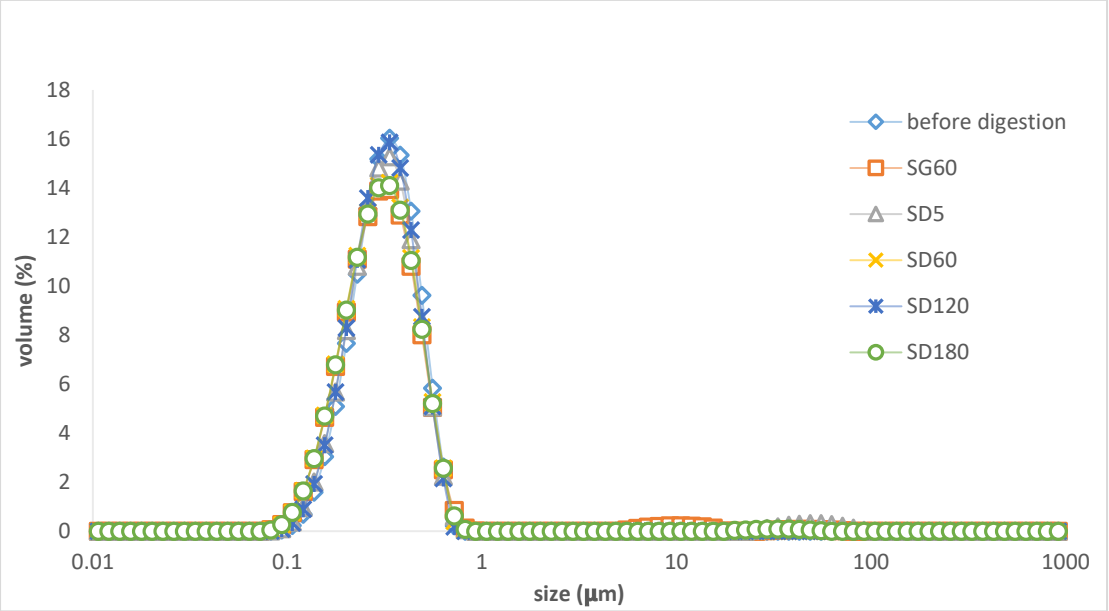

Figure 20 Droplet size distribution of nanoemulsion during digestion without enzyme. (Pepsin, pyrogallol, bile extract, phospholipids, pancreatic lipase, pancreatin) at gastric pH 1.6 (SG60: 60 min of gastric digestion; duodenal pH 6.8, SD5: 5 min of duodenal digestion; SD60: 60 min of duodenal digestion; SD120: 120 min of duodenal digestion; SD180: 180 min of duodenal digestion).

Figure 20 shows the droplet size distribution of nanoemulsion during *In vitro* digestion using fluids (SGF, SBF and SDF) without adding Pepsin, pyrogallol, bile extract, phospholipids,

pancreatic lipase and pancreatin. The results shown there is no significant difference in the drop size distribution of nanoemulsion (LTN) during each stage, which means fluids with varied pH from 1.6 to 6.8 had no effective on nanoemulsion stability.

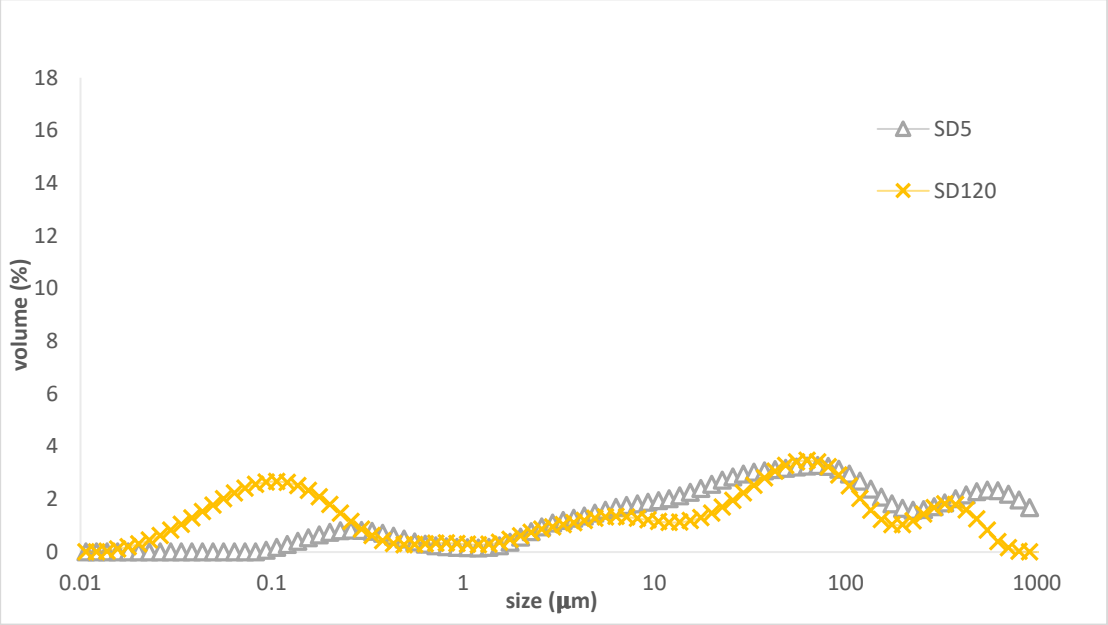

Figure 21 Droplet size of water during digestion with gastric pH 1.6 to duodenal pH 6.8 (SD5: 5 min of duodenal digestion; SD120: 120 min of duodenal digestion)

In contrast, figure 21 shows water as the control sample in the digestion model with pepsin, pyrogallol, bile extract, phospholipids, pancreatic lipase and pancreatin. There is no obvious peak shown in the figure 21. At the duodenal phase 5 min, particles size are in the range of above 0.1 µm, whilst, at the duodenal phase 120 min, some droplet were reduced to around 0.1 µm. Therefore, the digestive fluid containing phospholipids can help enzymes to hydrolyse lipids to fatty acids.

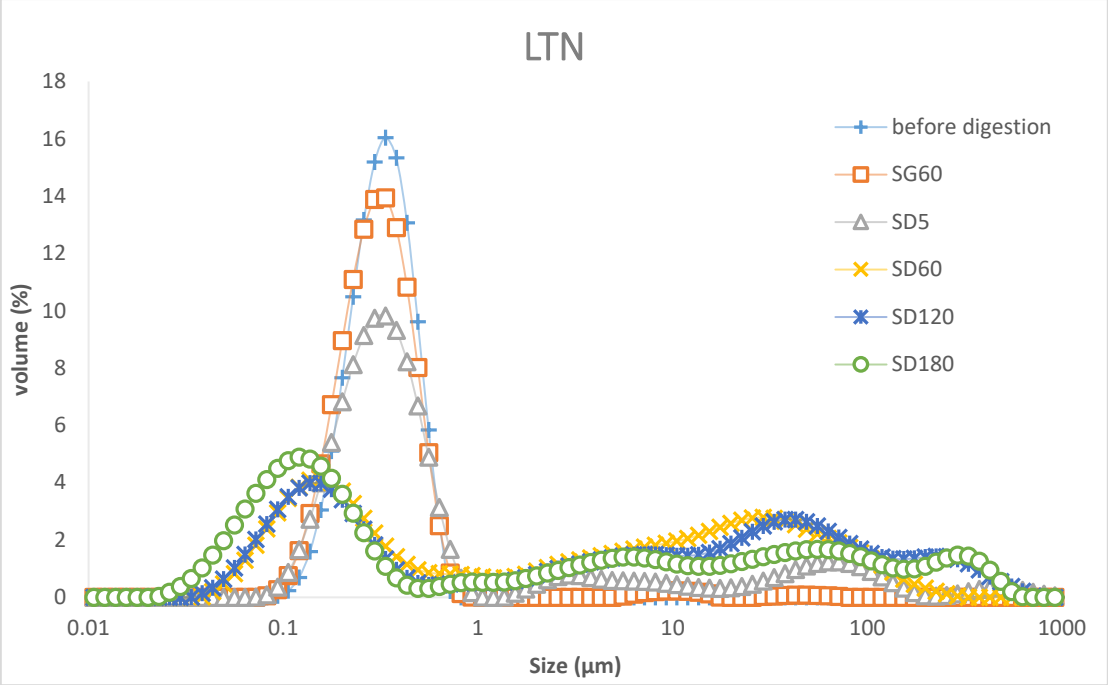

Figure 22 Droplet size of nanoemulsion LTN during digestion with gastric pH 1.6. (SG60: 60 min of gastric digestion; duodenal pH 6.8, SD5: 5 min of duodenal digestion; SD60: 60 min of duodenal digestion; SD120: 120 min of duodenal digestion; SD180: 180 min of duodenal digestion)

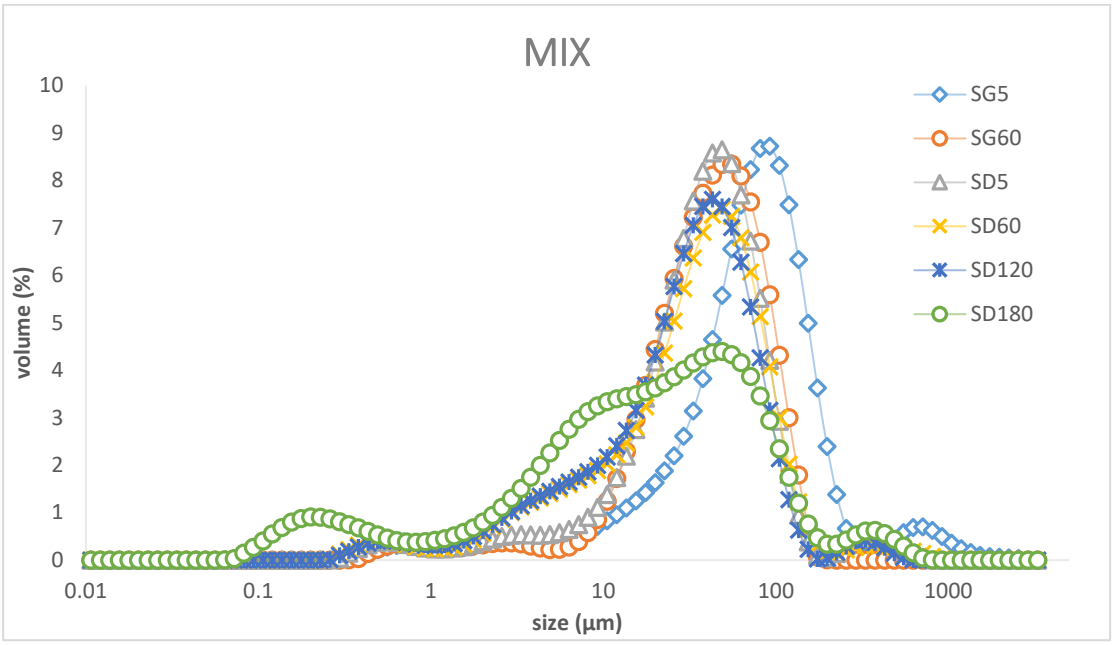

Figure 23 Droplet size of MIX during digestion with gastric pH 1.6. (SG60: 60 min of gastric digestion; duodenal pH 6.8, SD5: 5 min of duodenal digestion; SD60: 60 min of duodenal digestion; SD120: 120 min of duodenal digestion; SD180: 180 min of duodenal digestion)

Compare to figure 20, figure 22 shows the emulsion sample used fluids with added pepsin, pyrogallol, bile extract, phospholipids, pancreatic lipase and pancreatin. The results of the peaks show the emulsion had significant change during each stage of digestion. That means pepsin, pyrogallol, bile extract, phospholipids, pancreatic lipase and pancreatin have a large level of efficiency to digest emulsion samples.

After digestion, the samples were using ultracentrifuge for separation undigested triglycerides from algal oil were present in the aqueous phase sample. The digested sample of LTN was clearly separated (Figure. 24).

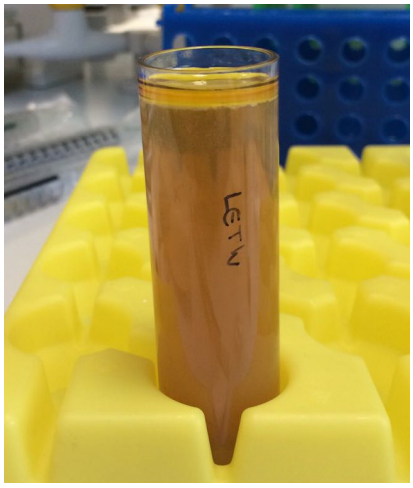

Figure 24 Appearance of digested LTN after ultracentrifugation

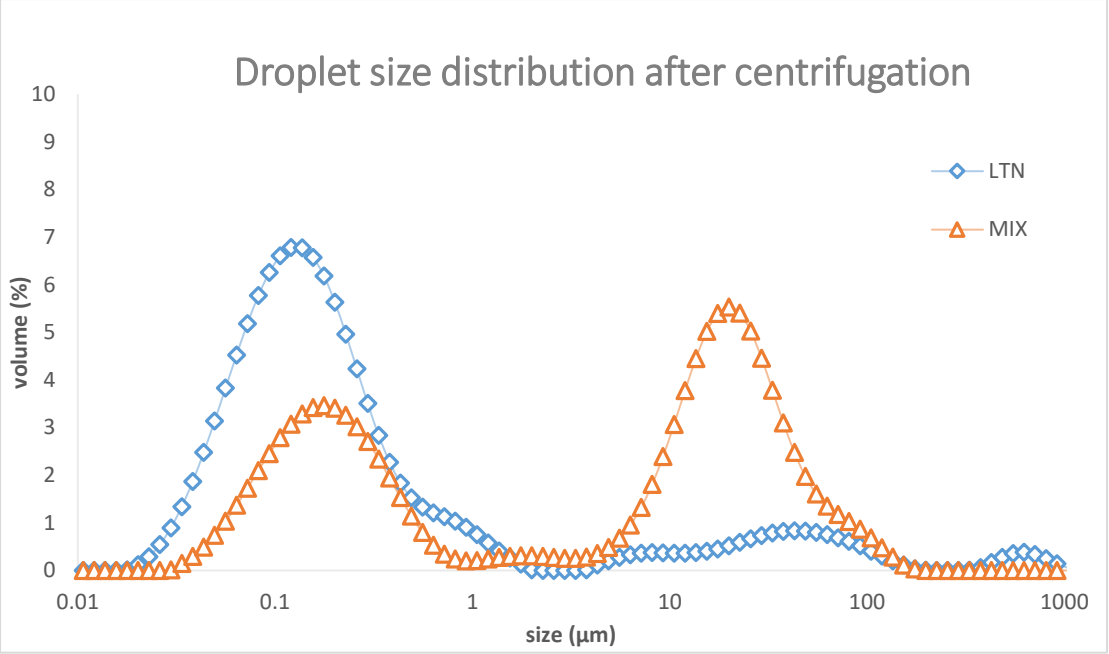

Figure 25 Droplet size of aqueous phase from digested LTN and MIX after ultracentrifuge.

The droplet size distribution of the aqueous phase is shown in Figure 25. One big peak in the range of 0.01 to 0.1 µm is observed for aqueous phase from digested LTN, showing an average drop size of 0.10 ± 0.002 µm. Contrast, two distinct peaks, one is in the range of 0.001 to 0.1 µm and another one in the range of 10-100 µm appeared in the droplet size distribution in the aqueous phase from digested MIX, demonstrating an average droplet size of 0.31 ± 0.006 µm, which is significantly larger than that from aqueous phase from digested LTN ($P < 0.05$). In the principle, the free fatty acids and monoglyceride released from the digestion will form a droplet of ~ 0.1 µm. Therefore, the larger peak in the range of 0.001 to 0.1 µm for aqueous phase from the digested LTN compared with that from digested MIX indicates that the LTN sample has a better digestibility than the MIX sample.

4.3.2 The digestibility of DHA of algal oil nanoemulsion in *In vitro* digestion model

The fatty acid composition of the algal oil and its nanoemulsion was analysed by gas chromatography and the DHA content of the aqueous phase from digested LTN and MIX were determined. Figure 26 shows the fatty acid esters from the algal oil nanoemulsion identified by gas chromatogram, which are identified as 14:00 myristic acid (14:00); palmitic acid (16:00); oleic acid (18:1n-9); linoleic acid (18:2n-6); osbond acid (22:5n-6); docosahexaenoic acid (DHA 22:6n-3) according to their retention time.

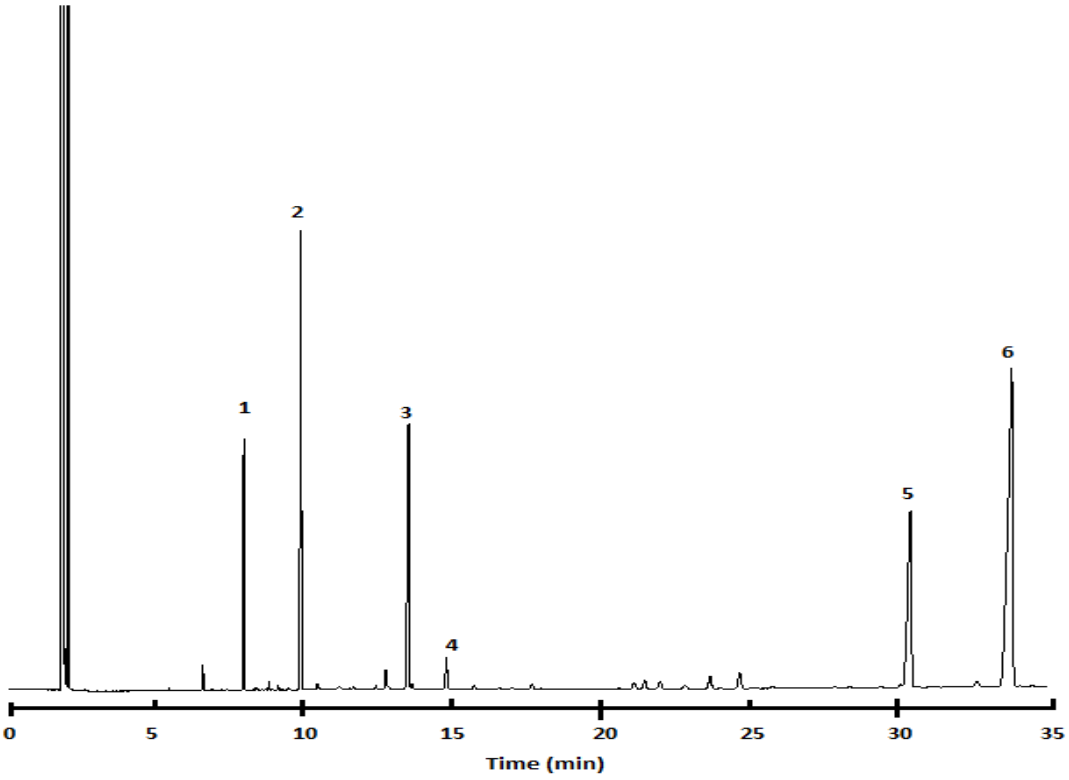

Figure 26 . Peaks of Fatty Acid Composition from algal oil nanoemulsion.(1. 14:00 myristic acid; 2. 16:00 palmitic acid; 3. 18:1*n-9* oleic acid; 4. 18:2*n-6* linoleic acid; 5. 22:5*n-6* osbond acid; 6. 22:6*n-3* docosahexaenoic acid (DHA).

In addition, the percentage of six fatty acids of algal oil nanoemulsiton and MIX (LTN) before and after digestion is shown in Table 10. It has been found that, the proportion of DHA in the algal oil nanoemulsion before digestion is significantly higher than in the

aqueous phase of the digested algal oil nanoemulsion. This result shows 73% DHA of algal oil nanoemulsion was transferred into aqueous phase after digestion. It is more interesting to find that the proportion of DHA from aqueous phase of digested algal oil nanoemulsion is much higher than that from aqueous phase of digested algal oil MIX digested under the same conditions, which is about 50% digested.

Table 10 The percentage of main fatty acids of LTN, aqueous phase of digested LTN and aqueous phase of digested MIX. Measured data are the means ± SD of duplicate lipid extraction from duplicate digestions.

%	14:00	16:00	18:1n-9	18:2n-6	22:5n-6	22:6n-3
LTN AND MIX before digestion	5.39±0.68	17.12±2.33	16.37±2.14	4.98±0.71	16.74±1.89	39.40±4.15
Aqueous phase of digested LTN	3.53±0.04	11.15±0.48	28.17±0.57	12.53±0.32	15.74±0.58	28.89±0.74
Aqueous phase of digested MIX	5.83±0.20	19.06±0.53	29.38±0.62	15.34±0.36	10.46±0.39	19.95±0.40

The DHA transferred into the aqueous phase of digested algal oil nanoemulsion and MIX was quantified using the DHA standard curve (Figure 27). LTN had a high incorporation (i.e.

73.32 ± 0.18%). Within LTN and MIX transfer in the aqueous phase was significantly different ($P < 0.05$).

After calculation, the DHA in LTN before digestion and the DHA in aqueous phase of LTN and MIX after digestion were presented in the table 10. It is clearly there is over 35% DHA including in the algal oil. After formulation, the DHA content of the LTN sample was 32%. Basic on the original algal oil composition determination, there is over 35% DHA including in the algal oil has been present on the label. After formulation, there is 16.2% DHA has been detected from the LTN sample (contain 50% algal oil). For the digested samples, the digestibility of DHA fatty acid from LTN after digestion is 28.1% of initial LTN, compared to the MIX sample after digestion which is 9.38% of initial LTN, it is significantly difference ($P < 0.05$). The digestibility of the LTN sample is two times more compare to the MIX sample.

Figure 27 Standard curve of DHA in a range of 0-200 µg/ml).

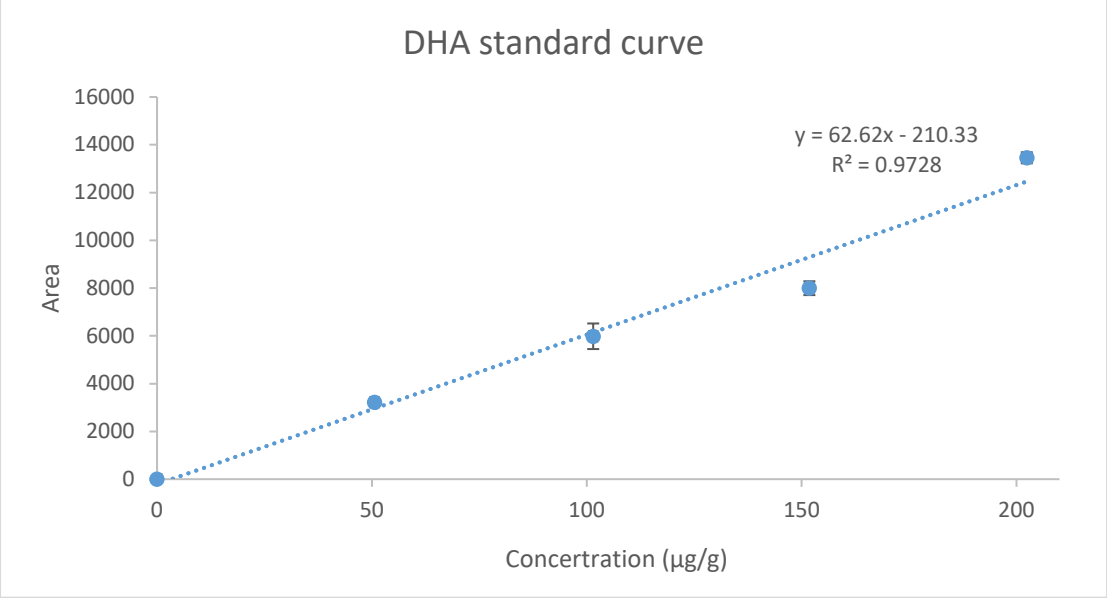

Table 11 The concentration of DHA in LTN before digestion and DHA of LTN and MIX after digestion released into aqueous phase.

	DHA/LTN and MIX (mg/g)	Digested DHA/LTN (mg/g)	Digested DHA/MIX (mg/g)
DHA	162.12±17.07	47.34±3.14	16.53±0.45

The DHA content in LTN and MIX, in aqueous phase of digested LTN and MIX were determined with DHA standard curve shown in the Figure 27. The results show that the DHA content in LTN or MIX is 162.12±17.07 mg/g. However, only 16.53±0.45 mg/g out of 162.12±17.07 mg/g was digested and transferred into aqueous phase of digested MIX, indicating only DHA of MIX digestibility is only 10%. Interestingly, 47.34±3.14 mg DHA was digested and transferred into aqueous phase of digested LTN, which clearly indicates that the DHA digestibility in LTN is about 3 times of DHA digestibility in MIX.

4.3.3 Discussion

In vitro digestion models have been extensively used to monitor the digestibility of food components and drug components under simulated gastrointestinal conditions (Hur *et al.*, 2010). The enzyme type, enzyme concentrations and food characteristics are key factors which control the digestion of fatty acids. In addition, the concentration of pancreatin lipase and bile salt play a key role in the triacylglycerol hydrolysis and formation of mixed particles into micelles for easy absorption by the intestine. In previous studies (Armand *et al.*, 1992; Lundin *et al*., 2008), it was found that when the droplet size of emulsified lipid decreased, the digestion rate of lipids increased, because the surface area of lipid exposed to digestive enzymes increased as the droplet size decreased. More recently, Lin *et al*., (2014) showed that algal nanoemulsions have different sizes at pH 1.6 and at 4.0, which is consistent with the observed effect that soy lecithin can reduce the droplet size of algal emulsions under acidic conditions during homogenization. The soy lecithin-stabilized emulsion phase separates when exposed to acidic gastric conditions (Mantovani, R. A. *et al*, 2013). At values close to the pKa of the anionic phosphate group (~ pH 1.5) of soy lecithin, the negatively charged phosphate group which at other pH values would generate electrostatic repulsion is shielded by hydrogen ions, thus providing conditions favourable for flocculation and coalescence (Comas *et al*., 2011). The emulsion was observed to rupture and release free oil during gastric treatment at pH 1.6. When acidic conditions are induced, emulsion flocculation appears to occur very rapidly, resulting in a large amount of coalescence and eventually rupture of the emulsion. Notably, emulsion destabilization was also observed when the pH of LTN was adjusted to pH 1.6 in the absence of SGF and pepsin, indicating that acidity is a key factor in emulsion destabilization. Preliminary experiments have shown that a slight increase in the droplet size of LTN at pH 4.0 is mainly due to the presence of SGF salts, which, when added separately, results in a significant increase in peak position.

In the current study, the LTN droplet sizes recorded in this study ranged from 0.01 to 1 μm (Figure 20). After the digestion, the droplet size of aqueous phase from digested LTN and from digested MIX and is significantly different (Figure 25). The droplet size in the aqueous phase of digested LTN, has more micelles of around 0.1 μm, whilst the droplet size in the aqueous phase of digested MIX has two separate peaks, respective located in the range of 0.15 μm and 17 μm. Large oil droplets of around 17 μm could not be possibly to be transferred into the blood stream without forming micelles (Lane *et al*., 2014). This indicates that every size oil droplet has to form micelles in order to be absorbed and hence smaller micelles are more easily adsorbed.

The previous study from Lin *et al*. (2014), DHA is transferred to the aqueous phase will affected by the pH, LTN treated with gastric pH 4.0 had higher DHA incorporation (ie, 62.49 ± 2.14%). Although the LTN treated with the stomach at pH 4.0 was finally hydrolysed to the same extent as the other samples, its DHA transfer was higher. This indicates that emulsification with lecithin promoted the transfer of DHA into the aqueous phase and was consistent with the higher bioavailability of LC3PUFAs from emulsified algal oil in human trials (Lane *et al*., 2014). Similarly, in a study of a linseed oil emulsion stabilized with soy lecithin rich in alpha-linoleic acid (ALA), it was found that emulsification improved the ALA concentration in rat lymph, although *In vitro* digestion test results indicated the amount of FFA released is low (Couëdelo *et al*., 2011). Subsequent measurements of lipid dissolution by bile salts showed that the emulsion facilitated transfer, although the emulsion hydrolysis was lower. In combination with the study, this indicates that the emulsification does play a role in enhancing the transfer of fatty acids from the oil to the aqueous phase. Although the LTN treated with the stomach at pH 4.0 was finally hydrolysed to the same extent as the other samples, which was stable at the beginning of duodenal digestion. This indicates that

emulsification with soy lecithin promoted the transfer of DHA into the aqueous. A higher DHA transfer at gastric pH 4.0 may be associated with a larger interface present at the onset of duodenal digestion, allowing relatively high LCPUFA exposure to digestive lipase.

In this study, DHA is transferred to the aqueous phase based on weight. LTN treated with gastric pH 1.6 had high DHA transfer is 73.32 ± 0.18%. In the MIX sample, DHA transfer in the aqueous phase is usually affected by gastric pH, DHA transfer was 50.63 ± 0.10% of the initial MIX sample. In this study, the final hydrolysis of DHA transfer with LTN treated with pH 1.6 was also very high. This indicates that the combination of soy lecithin and TWEEN 40 can stabilize the emulsification in a lower pH environment and better promote the transfer of DHA into the aqueous phase, in which the bioavailability is higher than the previous study Lin *et al.* (2014).

Additionally, the DHA transfer into aqueous phase has been quantified and results showed in Table 4 that for every 1 gram LTN, it contains 162.12 mg DHA, during digestion, 47.34 mg DHA was digested and transferred in to aqueous phase, differently, only 16.53 mg DHA was digested and transferred in to aqueous phase from MIX. It has been found that digestibility of DHA was increased from 10% for MIX to 30% for LTN, showing the DHA of algal oil has a very low digestibility. LTN developed in this study is efficient delivery system to improve the digestibility, in turn, enhance the bioavailability. For the FFA omega-3 consumption recommendation by EFSA Journal that 5.3 g algal oil nanoemulsion daily intake will maintenance human health beneficial (EFSA Journal DHA/EPA related health claims, 2011).

4.4 Conclusion

This study examined the digestion stability and digestibility of algal oil nanoemulsion (LTN) and MIX using an *In vitro* digestion approach. First of all, in this study, it was observed that the nanoemulsion LTN has been stabilized with combined emulsifier LE/TW at a ratio of 50:50 and the drop size of nanoemulsion stabilized at 267nm, there are around of 50% drop size is less than 200 nm. Secondly, this was found that it is stable with any destabilizing during the *In vitro* digestion with gastric phase at pH 1.6 for 60 mines. However, the droplets size of nanoemulsion in the duodenal phase at pH 6.8 was not stable in the same range as in the gastric phase due to the addition of the bile salt and pancreatic lipase and DHA digestion from droplets of nanoemulsion to micelles. Thirdly, the droplets of aqueous phase from digested sample after ultracentrifuge had been determined, showing that aqueous phase of nanoemulsion sample had a droplets of 0.10 ± 0.002 μm which is much smaller than that from MIX sample. Finally, the quantification of the aqueous phase DHA from digested samples by GC clearly the digestibility of nanoemulsion is much higher than the oil sample and emulsifier mix sample.

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Chapter 5 Oxidation within long chain omega-3 fatty acids of algal oil nanoemulsions

5.1 Introduction

Numerous short and long term studies have demonstrated that consumption of long chain omega-3 polyunsaturated fatty acids (LC3PUFA) offers benefits throughout the lifecycle for health promotion and a reduction in disease risk (Bowen, Harris, & Kris-Etherton, 2016; Shahidi, 2015). Oily fish is the preferred source for its high content of what are thought to be the most beneficial forms of LC3PUFA, eicosapentaenoic acid (20:5 ω3; EPA) and docosahexaenoic acid (22:6 ω3; DHA) (Lenihan-Geels & Bishop, 2016; Shahidi, 2015; Sun, Simonyi, Fritsche, Chuang, Hannink, Gu, *et al.*, 2017). However, oily fish may be unsuitable for population groups such as vegetarians/vegans (Papanikolaou, Brooks, Reider, & Fulgoni, 2014; Roberts, Steer, Mablethorpe, Cox, Meadows, Nicholson, *et al.*, 2018). The consumption in Western regions amongst the general population remains below recommended levels (Kranz, Jones, & Monsivais, 2017; Meyer, 2011). New approaches, including food based strategies have the potential to improve intakes and LCω3PUFA enriched functional foods may offer an alternative solution for this problem (Lane & Derbyshire, 2017; Papanikolaou, Brooks, Reider, & Fulgoni, 2014). The European Food Safety Agency (2011) have approved health claims in relation to foods naturally rich or fortified with EPA and DHA. Microalgal oils have recently emerged as a sustainable alternative source of DHA that is also suitable for vegetarians and vegans (Lane, Derbyshire, Li, & Brennan, 2014; Ryan & Symington, 2015; Sprague, Betancor, & Tocher, 2017).

Oxidation in fats and oils occurs because the lipids contain unsaturated acyl groups which are readily oxidised when in contact with oxygen producing unpleasant odours, bitterness and

other undesirable tastes and some toxic and harmful compounds. First, the oil produces hydrogenated oxides in the presence of oxygen in the air. The hydrogenated oxides, which are odourless and extremely unstable, are the main initial products of lipid oxidation. The hydrogenated oxide then further decomposes into aldehydes, ketones, acids, and other difunctional oxides, producing an unacceptable odour does not lead to rancidity. It is rancidity which leads to the unacceptable odour. Small molecule produced by oxidation of fats and oils can also be further polymerized to form dimers or multimers. For example, the oxidation product of linoleic acid is hexanal. The linoleate has a pentadiene structure, and the subunit at the 11th position is adjacent to the two double bonds, and is sensitive to oxidation. Even at 0° or lower, linoleic acid can be oxidized (Frankel E. 2010).

In previous work, high DHA vegetative algal oil load of 50% (w/w) was successfully used to establish an oil-in-water nanoemulsion system suitable for functional food enrichment. Nanoemulsion systems having a droplet size in the range of 20-500 nm have an improved bioavailability. Droplets in the range 20-500 nm are produced using ultrasound processing (Lane *et al*.2014). However, the oxidation stability of LC3PUFA nanoemulsion prepared using ultrasound technology may be a challenge for the application of LC3PUFA nanoemulsion in foods.

The aim of this study was to investigate the oxidative stability of nanoemulsions containing algal oils rich in docosahexaenoic acid (22:6n-3; DHA), and with a bulk oil under the same composition ranges of temperatures and storage times using gas chromatography headspace analysis (GCHS), gas chromatography (GC) and droplet size analysis.

5.2 Materials and Method

5.2.1 Materials

Algal oil Life DHATM S35-O300, was purchased from DSM Ltd., (Columbia, USA). L-α-Phosphatidylcholine (P3644-100G) of soybean (Lecithin), Type IV-S. ≥30% (enzymatic), Polyoxyethylenesorbitan monopalmitate (Tween 40, P1504-500ML) were purchased from Sigma-Aldrich, UK. Sodium chloride (99.5%) was purchased from ACROS, Spain. Hexane (HPLC Grade) was purchased from Fisher Scientific, UK. Methanol (HPLC Grade), Sulphuric acid 95%. Sodium sulphate anhydrous were purchased from VER BDH PROLABO chemicals, EC.

5.2.2 Preparation of Emulsion samples

Nanoemulsions of LCω3PUFA algal oil were prepared by following the method developed by Lane, Li, Smith and Derbyshire (2016), in which selected 6% (w/w) emulsifiers, i.e. lecithin, Tween 40, and equal ratio Tween 40 and lecithin, 50% (w/w) algal oil and 44% (w/w) water were used. A solution of 70% (w/w) algal oil mixed with 30% (w/w) lecithin was prepared two hours in advance and placed in a water bath at 55°C for 2 hours to ensure the lecithin was completely dissolved. Tween 40 was introduced directly into deionised water which had been bought to 55°C in a water bath. After premixing, appropriate measures of oil and water were added and the emulsions were replaced in the water bath for a further 2 hours and hand stirred for 1 min at 30 min intervals. The coarse emulsion sample was homogenized for two minutes at a speed (1200 rpm) by a homogenizer (Silverson Machine Ltd, England), then processed under an ultrasonic processor (BSP-1200 Ultrasonic processor, New York, USA) using Amplitude 100% with power 850w, operated at 19650Hz for 10 minutes to create nanoemulsions.

5.2.3 Measurement of droplet size

Nanoemulsions are classed as systems with droplet sizes ranging from 50 to 500nm (Kentish, Wooster, Ashokkumar, Balachandran, Mawson, & Simons, 2008; Sun, Xia, Zheng, Qiu, Zhang, McClements, *et al*., 2015) The droplet size of emulsion samples prepared was determined by Mastersizer 3000 laser light-scattering analyzer (Malvern Instruments Ltd, Malvern, UK) with a small sample dispersion unit set 2400 rpm. For the emulsion samples, an absorption parameter value of 0.001 was selected and a refractive index ratio 1.488 for algal oil (Lane, Li, Smith, & Derbyshire, 2016). For the purposes of this study, Sauter mean ($d_{32} = \sum n_i d_i^3 / \sum n_i d_i^2$ (Horiba Scientific, 2010)) has been reported as it reflects the surface diameter average value and the droplet size distribution and has been used in a number of previous studies (Lane, Li, Smith, & Derbyshire, 2016; Yang, Leser, Sher, & McClements, 2013).

5.2.4 Determination of fatty acid composition by GC

The fatty acid composition analysis of algal oil and nanoemulsion samples was performed by following a method developed from the FAME analysis method by the NoWFood research centre (2015, Unpublished results). 0.5 000 g algal oil /1.000 g of nanoemulsion sample and 10 ml Reagent A (2.5% w/v KOH solution in Methanol) were added into a MARSXpress vessel microwave digestion tube, then closed. The tube was placed into the Kevlar sleeves of a Mars 6 microwave (CEM Ltd., UK). Then, the temperature was increased to 90°C in 5 min and held for 10 min. After cooling to room temperature, 15ml reagent B (2% sulphuric acid v/v in Methanol) was added to the tube. After closing, the tube was placed back into the Kevlar sleeves of the Mars 6 microwave and the temperature was increased to 120 °C for 6 min. After cooling to room temperature, 10 ml Hexane was added to the tube and inverted once. The sufficient saturated salt solution was added to bring the hexane layer to the top

layer. The upper hexane layer containing fatty acid methyl esters was obtained for GC analysis using a GC Clarus 480. 200µl of the upper hexane layer containing fatty acid methyl esters and 800ul hexane was added into the GC vial with a small amount of added anhydrous sodium sulphate. The samples were analysed by GC Clarus 480 system (PerkinElmer Inc, USA) equipped with an auto sampler, Flame Ionization Detector (FID), 30 m, 0.25mm id 0.25 µm film thickness GC capillary column (SGE Analytical Science Pty Ltd, Australia) and Total Chrom Navigator software system (Version 6.3.2 PerkinElmer Inc, USA). The injector and detector temperature were 220°C and 250°C respectively, 1.5 µl of sample was injected in each time and hydrogen flow rate was set at 8.4 psi. The temperature program for the column was increased from 60 to 170°C at a rate of 20°C/min and to 200°C at a rate 1 °C/min, holding 1 min; the total run time was 36.5 min. Fatty acids were identified by reference to the retention time of standards. Analysis was performed in triplicate on individual vials for each time point.

5.2.5 Lipid oxidation compound analysis: GC Headspace Analysis (GCHS)

Gas chromatography (GC) was performed using 2g nanoemulsion samples prepared with 1 ml 1% NaCl solution, added to HS vial and vortexed for 30 secs. The samples were heated in a Headspace (HS) sampler (TuborMatrix 40 PerkinElmer Inc, USA) at 100°C for 60 min and injected under the following conditions: vial pressure 30psi; pressurise time 0.2 min; needle temp 100 °C; injection time 4.8 sec; withdrawal time 6 sec. The samples were analysed by HS GC Clarus 580, (PerkinElmer Inc, USA) equipped with a Flame Ionization Detector and 60 m 0.32 diameter column, 1.8 µm film thickness (Agilent Technologist, USA) under the conditions: Hydrogen flow rate 17 psi; injector temperature 230°C; detector temperature 230°C; oven temperature: from 40°C, ramp to 230°C at 20°C /min and hold at 230°C for 1 min, total time was 10.5 min. The volatile compounds were identified by reference to the

retention time of standards. Analysis was performed in triplicate on individual vials for each time point.

5.2.6 The storage trial

The tested samples were stored in the dark in incubators set at 4 °C, 20 °C and 40 °C for 5 weeks. The droplet sizes and volatile compounds were determined at baseline, week 1, week, 2 and week 5. Fatty acid composition was analysed for the bulk oil at baseline and bulk oil and nanoemulsions during week 1 and week 5 of the storage trial.

5.2.7 Experimental design and data analysis

All measurements were performed in triplicate. Results are expressed as mean ± standard deviation. Statistical analysis was completed using SPSS (v 24). Significant differences were identified ($p \leqslant 0.05$) by two-way analysis of Post-Hoc test (ANOVA) with a Tukey post hoc test at confidence intervals of 95%.

5.3 Results

5.3.1 The fatty acid composition of the algal bulk oil and its nanoemulsions

Changes in fatty acid profile were monitored at baseline and during the storage trial. The results are shown Fig 28 and Table 12. There are 6 main peaks for the fatty acids were identified on gas chromatogram of algal oil, which are 14:00 myristic acid; 2. 16:00 palmitic acid; 3. 18:1n-9 oleic acid; 4. 18:2n-6 linoleic acid; 5. 22:5n-6 osbond acid; 6. 22:6n-3 docosahexaenoic acid (DHA). Four unsaturated fatty acids are closely concerning the oxidation stability of algal bulk oil and its nanoemulsions.

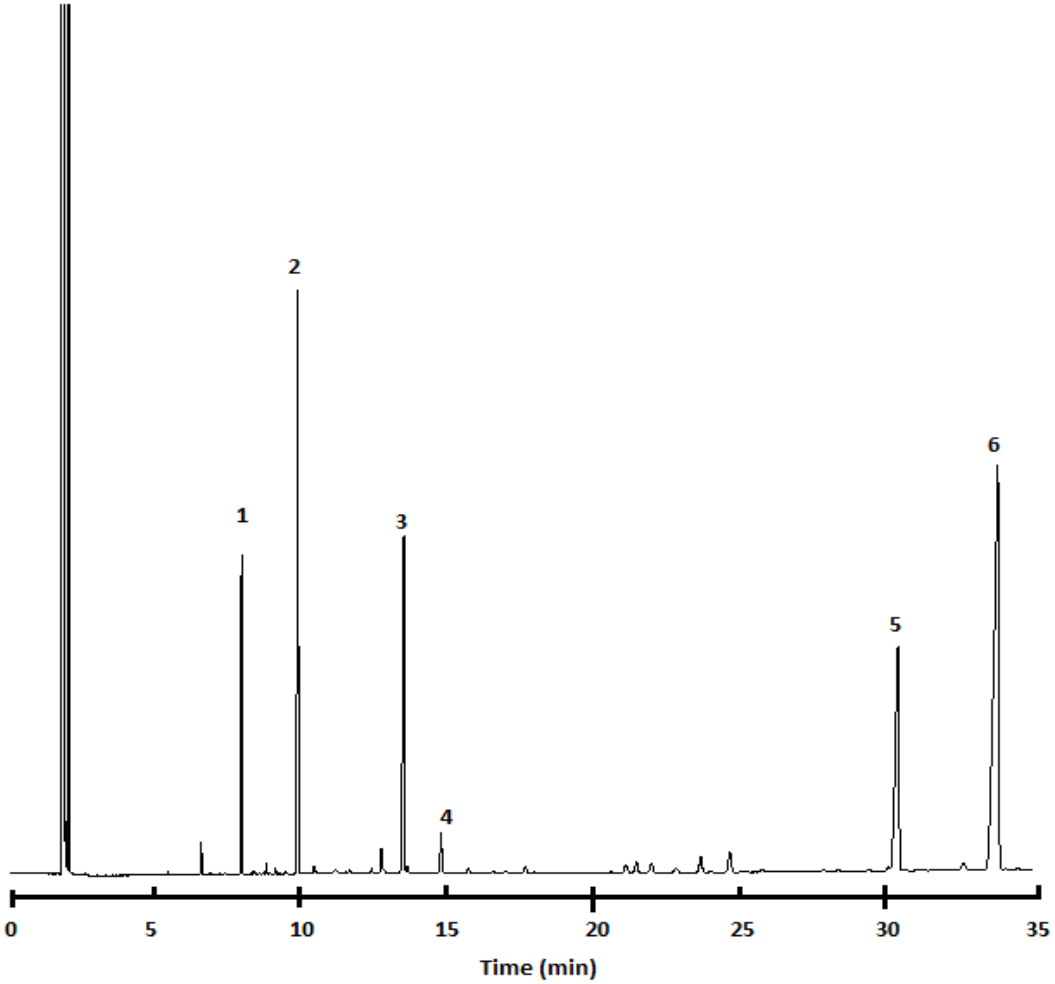

Figure 28 Gas chromatography of selected individual fatty acids of algal oil. (1. 14:00 myristic acid; 2. 16:00 palmitic acid; 3. 18:1n-9 oleic acid; 4. 18:2n-6 linoleic acid; 5. 22:5n-6 osbond acid; 6. 22:6n-3 docosahexaenoic acid (DHA))

Table 12 and Table 13 shows the percentile of 6 main fatty acids for algal oil and its nanoemulsion at the end of time of week 1 and week 5. It is noticed that 0.6% of linoleic acid and 42.74% DHA in the algal bulk oil. However, for LN and LTN, the percentile of linoleic acid increased to 6.61% and 4.98% respectively, whilst percentile of DHA dropped to 38.10 and 39.4% respectively, which indicates that lecithin contributed the linoleic acid to nanoemulsion, adding of factor to affect the nanoemulsion oxidation stability.

Table 12 The composition of 6 main fatty acids of algal oil and nanoemulsions at Week 1.

FFA Composition	14:0 %	16:0 %	18:1n-9 %	18:2n-6 %	22:5n-6 %	22:6n-3 %
4°C			week 1			
Oil	6.62±0.60	16.80±1.58	15.28±1.48	0.60±0.52	17.94±0.82	42.76±4.19
LN	5.99±0.91	17.53±2.71	16.15±2.65	6.68±1.10	16.42±2.82	37.24±7.81
LTN	5.23±0.20	17.26±1.13	16.59±1.29	5.09±0.39	16.59±1.03	39.03±2.43
TN	5.60±0.72	18.42±2.46	16.14±2.26	1.99±0.30	17.02±2.49	40.83±5.99
20°C			week 1			
Oil	6.46±0.48	16.67±0.75	15.38±0.63	0.90±0.05	17.84±1.35	42.76±3.26
LN	5.88±0.39	17.19±1.12	15.94±1.08	6.61±0.45	16.28±1.22	38.10±2.71
LTN	5.39±0.68	17.12±2.33	16.37±2.14	4.98±0.71	16.74±1.89	39.40±4.15
TN	5.60±0.49	18.40±1.63	16.16±1.48	2.00±0.19	17.05±1.63	40.80±3.81
40°C			week 1			
LN	5.91±0.41	17.21±1.21	15.96±1.14	6.66±0.48	16.27±1.20	37.99±2.72
LTN	5.40±0.47	17.27±1.51	16.53±1.53	5.00±0.48	16.63±1.63	39.18±4.15
TN	5.47±0.47	17.95±1.52	15.75±1.40	1.96±0.19	16.54±1.63	39.81±3.91

Table 13 The composition of selected individual fatty acid of algal oil and nanoemulsions at Week 5.

FFA Composition	14:0 %	16:0 %	18:1n-9 %	18:2n-6 %	22:5n-6 %	22:6n-3 %
4°C			week 5			
Oil	6.59±0.002	16.39±0.014	14.88±0.046	0.83±0.01	18.05±0.18	43.25±0.02
LN	5.91±0.50	17.36±1.53	16.24±1.48	6.81±0.62	16.66±1.56	37.03±5.77
LTN	5.27±0.03	16.92±0.05	16.34±0.11	4.97±0.03	16.82±0.56	39.67±1.21
TN	5.47±0.09	18.02±0.30	15.91±0.26	1.95±0.03	17.27±0.28	41.37±0.70
20°C			week 5			
Oil	6.58±0.35	16.45±0.88	15.03±0.83	0.84±0.06	17.56±0.27	43.54±2.38
LN	5.82±0.51	17.09±1.36	15.97±1.28	6.72±0.54	16.38±1.34	38.06±3.03
LTN	5.31±0.6	16.87±1.96	16.17±1.96	4.90±0.62	16.84±1.90	39.91±4.91
TN	5.52±0.26	18.17±0.94	15.98±0.89	1.95±0.11	17.25±1.04	41.12.±2.75
40°C			week 5			
Oil	6.71±0.10	17.70±0.12	14.07±0.10	1.39±0.05	17.66±0.09	42.47±0.07
LN	5.85±0.24	17.01±0.75	15.90±0.79	6.73±0.33	16.37±0.99	38.14±2.01
LTN	5.35±0.27	17.17±0.86	16.52±0.86	4.98±0.25	16.77±1.03	39.21±2.42
TN	5.42±0.34	18.03±1.05	15.95±0.92	1.94±0.14	17.27±1.15	41.38±2.93

Two-way ANOVA was used to analyse the changes in percentile of 6 main fatty acids during 5 weeks storage time. The results revealed that no significant differences in the percentage of DHA, other unsaturated fatty acids for sample, temperature and storage period, indicating that there was no sign of unsaturated fatty acid degradation in samples throughout the storage trial or minor degradation in samples was not detected with this method.

5.3.2 Nanoemulsion droplet size distribution

The droplet ranges of the nanoemulsions prepared with lecithin, Tween 40 and in combination can be found in Fig 29. At baseline the nanoemulsions prepared with Tween 40 (TN) and lecithin and Tween 40 combined (LTN) showed significantly smaller ($p \leq 0.05$) droplet sizes (242 ± 0.002 nm and 172 ± 0.002 nm respectively) than those prepared with

lecithin alone (LN) (340 ± 0.001 nm). 2-way ANOVA analysis revealed the lecithin samples had larger droplet ranges throughout the five weeks storage period at all temperatures ($p \leq 0.05$) compared with LTN and TN. The combined LTN sample shows consistently larger droplet sizes from baseline and 5 weeks at 20 and 40°C ($p \leq 0.05$). It is interesting to observe that there was no Signiant change in drop size for LN, TN and LTN throughout the storage period time.

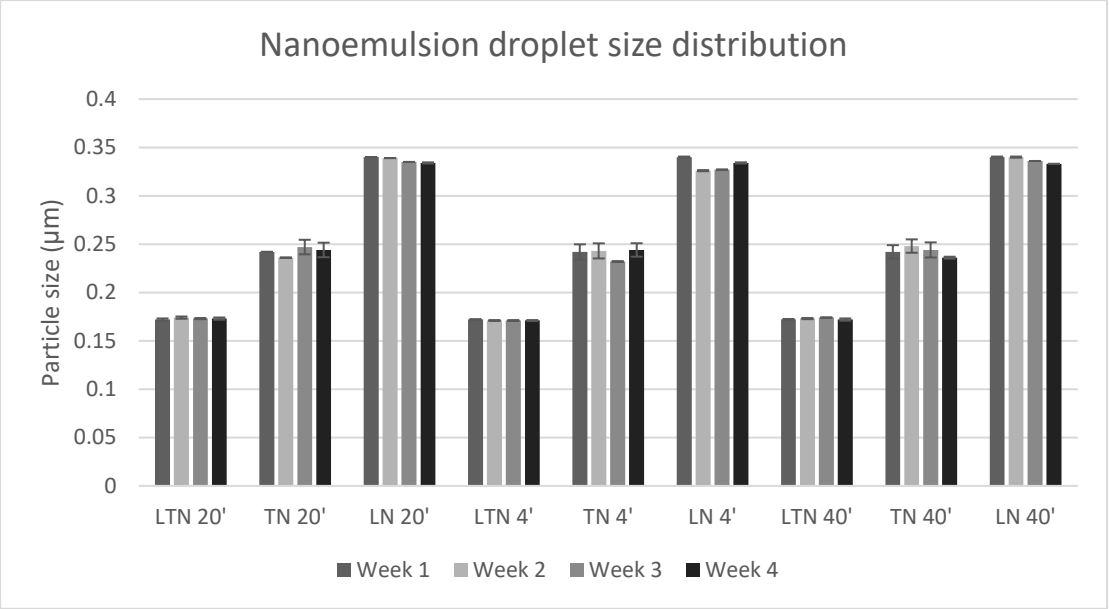

Figure 29 Droplet size distribution of nanoemulsion stabilized with different emulsifiers of Lecithin (LN), Tween 40 (TN) and Tween40/Lecithin (LTN) storage at 4, 20 and 40°C in 4 weeks.

5.3.3 Volatiles produced from the oxidation of algal oil and nanoemulsion during storage

HS-Gas chromatography was used to analyse the oxidised volatiles produced in algal oil and nanoemulsion during storage. Fig 30 shows that 5 main oxidised compounds, 1. Propanal; 2. 2-ethyl-furan; 3. Propan-3-ol; 4. Valeraldehyde; 5. Hexanal were identified and all of which have been associated with rancid off flavours in oxidised LC3PUFA oils and emulsions (Kolanowski, Jaworska, & Weißbrodt, 2007, Medina, I., Satué-Gracia, M. T. and Frankel, E.

N., 1999). The changes of those 5 oxidised compounds in tested samples were monitored during 5 weeks storage with varied temperatures. It was noticed that propanol has the largest peak in those five compounds at the week 1 for all the tested samples (Table14, 15, 16 and 17). Significant differences were noted for 2.2-ethy-furan, propan-3-ol in the nanoemulsions samples stabilised with lecithin (LN and LTN) stored at 40 °C after 2 weeks ($p \leqslant 0.05$).

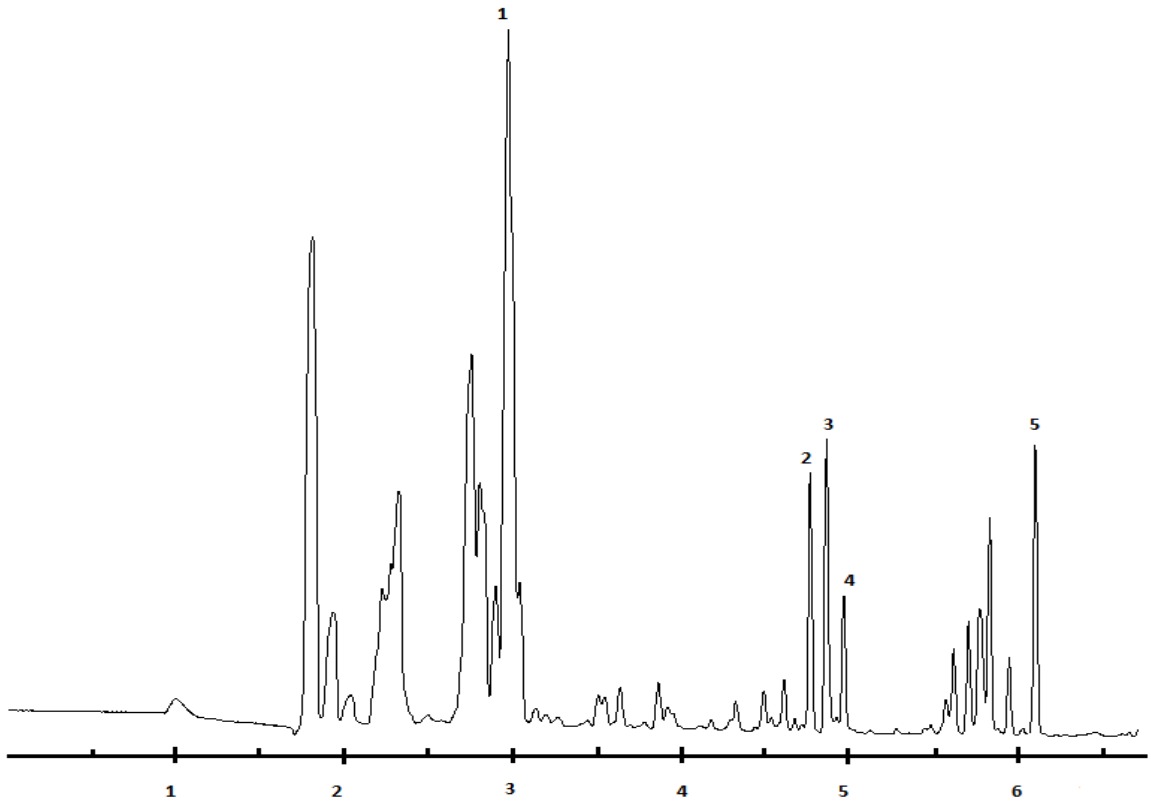

Figure 30 HS- Gas chromatogram of identified oxidised compounds produced by algal oil nanoemulsion (1. Propanal; 2. 2-ethyl-furan; 3. Propan-3-ol; 4. Valeraldehyde; 5. Hexanal)

Bulk oil sample had least amount of significant differences in volatile compounds when analysing storage time and temperature. The combined lecithin and Tween 40 sample showed less significant increase on volatile propanal, at 20 and 40 °C in than the lecithin only sample, however, the Tween 40 only sample had the significant differences in volatile compound hexanal, which also showed on combine lecithin and Tween 40 sample. LN also had significant increase on 2-ethylfuran and Propan-3-ol 20 and 40 °C from week 1 to week 5.

All samples at 4 °C remained the most stable with the least amount of significant differences in volatile compounds for this temperature. An increase in sample storage temperature results had a significant increase in all volatile compounds in all samples. The two lecithin samples showed more significant differences in the storage time and temperature of the volatile compounds than the Tween 40 sample at 40 °C, again indicating that Tween 40 provides overall protection and the stability of lecithin to oxidation was lower, especially at elevated temperatures.

Table 14 Oxidised compounds produced by bulk algal oil during storage at 4, 20 and 40°C determined using HS Gas chromatograph

Oil		Propanal	2-ethylfuran	Propan-3-ol	valeraldehyde	Hexanal
4°C						
	Baseline	24842.84±12532.9	1387.75±275.74	2568.99±1056.64	1283.49±359.95	4955.02±1715.53
	Week1	7651.52±155.56	1098.65±18.72	1313.03±410.92	995.36±17.85	3554.05±141.7316
	Week2	46504.11±32846.38	2829.28±2162.36	4592.7±2528.86	1994.81±1379.312	8622.653±5598.343
	Week5	7065±5153.3	1175.82±294.53	1138.81±315.74	1405.71±510.57	4293.56±1535.67
20°C						
	Baseline	24842.84±12532.9	1387.75±275.74	2568.99±1056.64	1283.49±359.95	4955.02±1715.53
	Week1	7651.52±155.56	1098.65±18.72	1313.03±410.92	995.36±17.85	3554.05±141.7316
	Week2	153570.1±57352.66[a, A]	4389.26±471.73[a]	46596.07±55108.23	9985.07±630.02[a,A]	15970.01±3777.689[a,A]
	Week5	87246.28±3926.33	3612.65±1356.67	10392.83±341.148	49968.2±143.44A	7888.1±546.86
40°C						
	Baseline	24842.84±12532.9	1387.75±275.74	2568.99±1056.64	1283.49±359.95	4955.02±1715.53
	Week1	7651.52±155.56	1098.65±18.72	1313.03±410.92	995.36±17.85	3554.05±141.7316
	Week2	119512.5±27131.02	2849.01±426.81	9660.44±2168.67	2067.2±249.78	11488.65±2193.952
	Week5	266506.2±98586[a, A]	4477.76±1780.1[a,A]	23729.58±8216.07	6799.61±2606.54[a,A]	22117.46±7463.08[a,A]

a: Indicates significant differences between storage times A: Indicates significant differences between storage temperatures.

Table 15 Oxidised compounds produced by algal oil nanoemulsion stabilized by 6% lecithin during storage at 4, 20 and 40°C determined using HS Gas chromatography

LN		Propanal	2-ethylfuran	Propan-3-ol	valeraldehyde	Hexanal
4°C						
	Baseline	44913.43±2201.79	5633.02±109.91	8219.32±739.1	16252.44±542	23936±1272.58
	Week1	102757.8±38255.58	13122.01±5034.23	16120.97±5264.99	19647.8±7193.4	36710.01±12734.02
	Week2	150174.5±6087.08	17934.31±999.31	22880.2±500.76a	21413.66±325.61	38513.04±1049.41
	Week5	110398.2±38228.17	13326.27±4708.24	17943.34±6589.57	18147.43±5840.73	22969.34±11116.63
20°C						
	Baseline	44913.43±2201.79	5633.02±109.91	8219.32±739.1	16252.44±542	23936±1272.58
	Week1	165865.9±489.95[a]	21705.4±2.1a	28419.67±14.04	25701.33±537.31	48808.01±913.16
	Week2	125430.1±44876.62	18629.73±5691.22[a]	20820.27±5797.19a	8800.83±2097.04[A]	23410.08±5567.83
	Week5	175276±46031.18[a]	25898.45±4130.9[a]	33016.13±3168.46a,A	13792.41±940.38	27012±6351.09
40°C						
	Baseline	44913.43±2201.79	5633.02±109.91	8219.32±739.1	16252.44±542	23936±1272.58
	Week1	306664.2±9482.03[a,A]	42303.26±1913.36[a,A]	50754.29±646.5482[a,A]	26405.99±409.49	57320.96±10437.13[a]
	Week2	203925.4±90123.4[a]	25053.8±7071.18[a]	29800.22±8537.95[a]	17485.64±5151.8	45195.6±12571.16
	Week5	263618±67145.2[a, A]	37470.81±9492.57[a,A]	31608.97±7707.56[a,A]	23443.53±5826.38	64734.87±19100.06[a,A]

a: Indicates significant differences between storage times A: Indicates significant differences between storage temperatures.

Table 16 Oxidised compounds produced by algal oil nanoemulsion stabilized by 6% Tween40 during storage at 4, 20 and 40°C determined using HS Gas chromatography

TN		Propanal	2-ethylfuran	Propan-3-ol	valeraldehyde	Hexanal
4°C						
	Baseline	13628.6±7430.75	3118.48±973.47	4041.46±1087.57	656.81±428.92	2806.53±341.86
	Week1	59698.09±5399.7	7086.56±912.67	7747.4±457.77	827.05±84.79	6972.97±2531.56
	Week2	161155.8±135887.8	10647.44±8348.11	24406.21±18926.6[a]	2903.84±2446.11	25234.2±20374.05
	Week5	161859.1±12819.23	21292.68±5622.96[a]	25182.85±2471.92[a]	2258.34±801.39	23734.18±2404.15
20°C						
	Baseline	13628.6±7430.75	3118.48±973.47	4041.46±1087.57	656.81±428.92	2806.53±341.86
	Week1	56054.94±33169.79	10958.07±4205.52	13996.41±5059.66	19418.58±6348.1[a,A]	31413.39±15573.24[a,A]
	Week2	226867.4±3241.5[a]	11095.24±1653.5	30264.8±4331.22[a]	5808.2±454.22	29578.12±3682.37[a]
	Week5	196127.3±32536.75[a]	21587.94±5484.66[a]	30095.91±3173.1[a]	7689.15±7150.04[a]	26600.65±3809.5[a]
40°C						
	Baseline	13628.6±7430.75	3118.48±973.47	4041.46±1087.57	656.81±428.92	2806.53±341.86
	Week1	107139.4±46935.58	10529.16±0	13292.6±0	1546.61±0	12863.85±0
	Week2	357994.4±85487.8[a]	13651.26±1502.88	48171.1±8224.61[a,A]	6445.38±1437.94	46258.49±10216.66[a]
	Week5	299928.4±16948.06[a,A]	11081.96±1008.79	40114.84±1651.08[a]	6603.94±6392.84	42513.74±996.14[a]

[a]: Indicates significant differences between storage times [A]: Indicates significant differences between storage temperatures.

Table 17 Oxidised compounds produced by algal oil nanoemulsion stabilized by 3% lecithin and 3% Tween40 during storage at 4, 20 and 40°C determined using HS Gas chromatography

LTN	**Propanal**	**2-ethylfuran**	**Propan-3-ol**	**valeraldehyde**	**Hexanal**
4°C					
Baseline	32084.01±0	5788.86±0	6687.58±0	3987.43±0	17492.75±0
Week1	59321.16±773.90	11090.92±946.22	12285.23±213.153	6675.55±209.16	30493.1±1120.35
Week2	140201.2±14239.99	18776.48±911.05	20814.22±2225.27	12078.74±8493.48	42121.75±2734.76[a]
Week5	130062.9±11497.58	22397.19±2709.64[a]	21114.93±1305.01	6196.08±310.59	42650.72±1211.56[a]
20°C					
Baseline	32084.01±0	5788.86±0	6687.58±0	3987.43±0	17492.75±0
Week1	57090.97±2770.84	11088.26±1736.78	12718.25±796.93	7098.87±284.421	30280.06±1323.57
Week2	67144.3±77958.1	24211.04±1348.41[a]	32967.4±6969.69[a]	10207.16±66.25	47101.22±17107.54[a]
Week5	209987.2±123433.36[a]	11319.99±6417.16	23787.33±12829.2[a]	4477.93±2468.12	19532.97±10698.34[A]
40°C					
Baseline	32084.01±0	5788.86±0	6687.58±0	3987.43±0	17492.75±0
Week1	71597.26±9864.61	213078.73±564.13	16354.94±549.44	7472.367±147.57	38350.93±461.43[a]
Week2	271886.1±42527.65[a]	19464.7±13876.99[a]	41249.95±10907.88[a,A]	9696.11±5875.53	80247.73±10421.71[a,A]
Week5	458613.7±17751.51[a, A]	41125.13±2800.66[a,A]	47851.73±2209.94[a,A]	18549.4±450.41[a,A]	109196.8±3445.581[a,A]

[a]: Indicates significant differences between storage times [A]: Indicates significant differences between storage temperature

5.4 Discussion

5.4.1 The fatty acid composition of the bulk oil and nanoemulsions

These findings are similar to previous research by Karthik and Anandharamakrishnan (2016b) who found no differences in fatty acid composition of bulk algal oil and Tween 40 nanoemulsions stored for 100 days. Systems were created using microfluidization and no loss of DHA was indicated during the emulsion process. The authors concluded there was no change in fatty acid profile and structural changes to DHA in any of the emulsions tested. Ghorbanzade, Jafari, Akhavan, & Hadavi (2017) found that DHA and EPA retention was maximised in purified fish oil and soy lecithin nano-liposomes created using ultrasound when added to yoghurt during 21 days of storage at 4°C. In contrast, analysis of fatty acid composition of bulk fish oil, sonicated fish oil and fish oil nanoemulsions created with whey protein isolate (WPI) by Nejadmansouri, Hosseini, Niakosari, Yousefi, & Golmakani (2016) showed significant decreases in EPA and DHA composition during a 1 month storage period. This indicates fish oil nanoemulsions stabilized with WPI may have lower oxidative stability compared to systems stabilised by lecithin. The results of this study demonstrate that there was no loss of DHA and that it remained stable for all storage conditions and emulsifiers, which is comparable to other work in the field.

5.4.2 Nanoemulsion droplet size distribution

The droplet size ranges of the emulsion samples were measured by laser light scattering particle sizer at baseline and various intervals during storage at the different temperatures. Variations in emulsion droplet size can impact oxidative stability due to differences in droplet surface area and the amount of light that can penetrate through the system. Smaller emulsion droplet size ranges lead to increases in droplet surface area and increased light penetration through the emulsion, which may reduce oxidative stability (Uluata, McClements, & Decker,

2016). Karthik & Anandharamakrishnan (2016a) also found soy lecithin produced nanoemulsions with larger droplet ranges in comparison to Tween 40 and sodium caseinate stabilised algal oil nanoemulsions created using high power microfluidization. The Lecithin only sample droplet ranges remained at similar higher ranges throughout storage at all temperatures. Uluata, McClements, & Decker (2016) found no differences as a function of particle size on auto oxidation rates in a similar fish oil nanoemulsion study. The results of this study show dropsize of nanoemulsions prepared using lecithin were consistently larger than LTN and TN during the storage period of time regardless the temperatures. In the tested nanoemulsion samples (LN, LTN and TN), TN the smallest droplet range and remain the most stable with droplet ranges unchanged at all temperatures and there were no significant changes in the drop size for all tested nanoemulsion.

5.4.3 Oxidation and volatiles produced in nanoemulsion preparation and storage

HS-GC is used widely to measure volatile compounds produced in the terminal stages of lipid oxidation and is beneficial as no oil extraction methods are required (Medina, I., Satué-Gracia, M. T. and Frankel, E. N., 1999). Hexanal and propanol have been previously identified as common indicators of secondary oxidation for LCω3PUFA nanoemulsions (Medina, I., Satué-Gracia, M. T. and Frankel, E. N., 1999, Sharif, Williams, Sharif, Khan, Majeed, Safdar, *et al*., 2017). In current study, another 3 oxidised compounds were identified, which has provided a better understanding on the oxidation process of omega 3 fatty acids.

The nanoemulsion sample with combined lecithin and Tween showed less significant increases in volatile products at week 1 than the lecithin only sample and the Tween 40 only sample had the least amount of significant differences in volatile compounds when analysing storage time and temperature. However, the Tween 40 samples show more significant

differences in the formation of hexanal in comparison to the lecithin only sample and this was further accelerated by increases in temperature. All samples remained more stable at 4°C with the least amount of significant differences in volatile compounds noted at this temperature. Increases in sample storage temperature led to significant increases in the development of volatile compounds for all samples. Both lecithin samples demonstrated more significant differences in volatile compounds for storage times and temperature than the Tween only samples at 40°C, again indicating that Tween 40 may offer an overall protective effect whilst lecithin is less stable to oxidation particularly at increased temperatures. This is comparable to research by Uluata, McClements, & Decker (2015) who found that fish oil and lecithin nanoemulsions had higher propanal development at increased temperatures.

The fatty acid composition of samples in this study showed no significant differences for temperature and storage times. However, suggests the oxidative stability of the lecithin itself rather than the oil may have affected the development of volatile compounds. It is possibly because the because the linoleic acid is in lecithin molecule and it would in the out layer of emulsion drops, closer to water phase (oxygen). In contrast, DHA of algal oil would be within the oil drop and be covered by the lecithin molecules. So in this case, the linoleic acid is less stable than DHA in this system. The droplet size range measurements showed the lecithin only samples had significantly larger droplet size ranges throughout the trial, which suggests that larger droplet size ranges do not offer a protective affect for the development of volatile compounds.

Karthik & Anandharamakrishnan (2016a) also found lecithin samples had larger droplet ranges and that Tween 40 could be used to create systems with smaller droplet ranges and offered a protective effect for oxidative stability. Recent studies have identified that the emulsifier quillaja saponin offers equivalent or enhanced oxidative stability in comparison to

other natural and synthetic emulsifiers (Bush, Stevenson, & Lane, 2017; Uluata, McClements, & Decker, 2015) and therefore could be used in substitution to lecithin. Further studies would therefore be beneficial to conduct in depth fatty acid composition, droplet measurements and GCHS analysis using different variations and compositions of samples containing, lecithin, Tween 40 and algal oils.

5.5 Conclusion

There were no significant differences in fatty acid composition for all samples throughout the storage trial at all temperatures. The drop size for all tested samples remained stable throughout the 5-week storage trial. The combined sample had significant increases in droplet range measurements at higher temperatures after 5-weeks of storage. The nanoemulsion systems with the sole and combined lecithin developed significantly more volatile compounds at higher temperatures. This would suggest that added lecithin may be one of causes to celebrate the oxidation in those two nanoemulsion samples, in which the increase in percentile of linoleic acid with added lecithin may a main factor. Samples created using Tween 40 had the smallest, most stable droplet ranges and developed fewer volatile compounds than the lecithin containing samples. This refutes the initial hypothesis demonstrating that Tween 40 produced the most stable systems in terms of physical and oxidative stability.

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Chapter 6 A Sensory evaluation on foods enriched with algal oil and its nanoemulsion

6.1 Introduction

Sensory science is “a scientific discipline used to evoke, measure, analyse, and interpret the response to food and other material characteristics because they are divided into visual, olfactory, tactile, taste, and auditory perceptions.” (Stone and Sidel, 2004; O'Mahony, M. 2017). Sensory evaluation is a key method for assessing the quality of food because it measures the true feelings of consumers and it is a subjective approach (Lawless & Meilgaard, M. C., Carr, B. T., & Civille, G. V. 1999; Lawless, H. T., & Heymann, H. 2010; O'Mahony, M. 2017). For example, one consumer may describe the sample as uncomfortable while another consumer may consider the same sample acceptable (Bryhni *et al.*, 2002). These differences are common in sensory evaluation and can be explained by nationality, culture and individual preferences of panellists. Therefore, in order to obtain an effective sensory evaluation, a reasonable number of panellists are required (Bi, J. 2015; Lawless, H. T., & Heymann, H. 2010). The sensory attributes used for testing need to be carefully designed based on the intrinsic characteristics of food substrate being tested and any possible variations between different samples caused by food formulation, processing, packaging, storage conditions need to be considered. Therefore, sensory evaluation is time-consuming and expensive testing (Bryhni *et al.*, 2002). However, compared with most analytical methods for measuring food quality require sample preparation, such as appropriate heating to measure volatile compounds of food sample by gas chromatography and solvent extraction to measure peroxide in the snack.

Although the delivery system of emulsion for Omega-3 has been studied in previous research (Kaemi *et al*., 2007; Jacobsen *et al.,* 2010), there are current problems in the stability, oxidation and sensory palatability that need to be solved (Moore *et al.*, 1998; Lesmes *et al.,* 2010; Haham *et al*., 2012; Lane, 2013).

In addition, Lesmes *et al*., (2010) found that the lipid oxidation of emulsion also affects flavour, texture, appearance, and nutritional quality of food products with omega-3 enriched foods.

A Previous study by Lane *et al.* (2013) showed nanoemulsion had a significantly negative effect on sensory acceptability, and demonstrated the sensory differences between the emulsions with different emulsifiers, for example, the nanoemulsions made with lecithin are better than Tween 40. The results from Moors *et al*., (1998) stated that sensory attributes were significantly affected by higher oil phase of emulsion. The droplets size of emulsion is known to affect the release of lipophilic flavours from the oil droplets, where increasing the diameter correlate to an increase in aroma release (Van Ruth *et al.*, 2002).

A comparison ranking testing to test sensory profile of samples, which has the advantage being able to test more than two samples in each run. In addition, a reference sample can be updated, providing the agreed standard for each attribute in testing. It was used in this study to compare the addition of bulk oil or nanoemulsion on the sensory profile of white sauce.

Participants were selected from the postgraduates and staff of University of Chester with a Food Science and Nutrition background and they were trained with basic knowledge of sensory evaluation and definition of sensory attributes.

In addition, since some external physical properties may have an impact on the test results as the test progresses (Stone and Sidel, 2004). In this study, a well-designed sensory booth of NowFOOD research centre was used to minimize the bias of team members.

Sample preparation in this study also required strict control conditions. The preparation area separated from the test chamber but close to the test chamber allows efficiently to prepare and provide test samples and clean them after the test is completed. Test samples were prepared and supplied under controlled conditions. Cooking time, service temperature, and the number and appearance of samples were also standardized to minimise the variation in the preparation.

6.1.1 Objectives

To conduct a primary sensory testing to evaluate the sensory profile of food products enriched algal and its nanoemulsion.

To explore possible relationship between sensory profile of white sauce and characteristics of nanoemulsion.

6.2 Method and Materials

6.2.1 Materials

Algal oil Life DHATM S35-O300 was purchased from DSM Ltd., (Columbia, USA). Soybean liquid lecithin, NOW Ltd, (USA), Polyoxyethylenesorbitan monopalmitate (Tween 40, P1504) were purchased from Sigma-Aldrich, UK.

6.2.2 Methods

6.2.2.1 Oil-in-water nanoemulsion preparation

The nanoemulsion of omega-3 fatty acids oil was prepared using Lane's (2013) method with selected 6% (w/w) of combination emulsifiers, including: lecithin, Tween 40, Tween 60 and equal ratio of Tween 40 and lecithin, and 50% (w/w) of algal oil and 44% (w/w) of water. The combination of lecithin and algal oil in 30:70 ratios was place in a water bath at 56 ºC for 2 hours. After premixing, extra algal oil and water was added in and was replaced in the water bath for additional 2 hours. It was stirred by hand for 30 seconds every hour. The coarse emulsion sample was homogenized for two minutes at a speed (1200 rpm) on homogenizer (Silverson Machine Ltd, England), then processed under an ultrasonic processor (BSP-1200 Ultrasonic processor, New York, USA) using Amplitude 100% with power 850w, operated at 19650Hz for 10 minutes to create nanoemulsions.

6.2.3 Sensory Testing

In this study, the Eyequestion software was used and ranking testing was selected to compare the sensory profile of white sauce with or without bulk oil/its nanoemulsion. White sauce were prepared using white béchamel sauce recipe from Sainsbury's supermarket Lasagne, which includes whole milk, double cream, single cream, butter crumb, parmegiamo cheese, starch, flour, white pepper, bay and salt. The White sauce were incorporated of algal oil and

omega-3 nanoemulsions to increase DHA content to 250mg and 2000mg per 100g of white sauce sample, thus 250mg DHA to maintain the normal eye, brain and heart health, and 2000mg to achieve maintain the normal blood lipid profile (EFSA, 2011). The samples were mixed using Thermomix TM5 (Vorwerk UK Limited, UK) under condition temperature 70 °C in 12 mins at mixing speed 1.

The 11 trained participants were invited from the University of Chester to have a sensory testing. The nine sensory attributes scores of the smooth samples, i.e. colour, cheese aroma, fish aroma, salty taste, fish taste, aftertaste, texture, mouth feeling and overall liking acceptability, data collected and analysed using EyeQuestion software.

Table 18 The DHA content in white sauce samples for sensory testing.

	Control	**Low level oil**	**High level oil**	**Low level emulsion**
White sauce (g)	100	100	100	100
Oil/ EM (g)	-	0.7	5.56	1.4
DHA conc. (mg)	-	250	2000	250

6.2.4 Statistical analysis

All data were collect by EyeQuestion software. Results are expressed as mean ± standard deviation. The analysis of post-hoc test (ANOVA, SPSS statistic v 24) was used to compare sample mean to check for significant differences in distribution of sensory testing data, and compere the all data from droplet size testing of nanoemulsion.

6.3 Results

6.3.1 Sensory properties of white sauce enriched with bulk oil and its nanoemulsion

The sensory testing (ranking testing) were also conducted on white sauce enriched with algal oil and its nanoemulsion. The sensory attributes of tested samples, cheese aroma, fish aroma, fish taste, aftertaste and overall liking were evaluated and the results are shown in Table 19 and Figure 31.

Table 19 Sensory profile of white sauce with and without algal oil and its nanoemulsion.

	Control (A)	Low level oil (B)	High level oil (C)	Low level emulsion (D)
colour	52.66±5.23	53.97±7.46	53.51±9.15	55.99±9.24
Cheese aroma	49.00±6.05	50.85±7.78c	37.94±15.50	39.96±7.60
Fish aroma	46.62±15.78	45.62±16.11	64.04±17.42$_{a}$	59.81±9.46
Salty Taste	48.17±11.30	53.90±6.24	56.21±10.96	56.29±13.05
Fish Taste	48.00±3.12	48.53±8.54	68.62±18.45$_{a}$	66.33±15.48
Aftertaste	51.29±5.99	53.65±8.65	64.75±12.46$_{ab}$	64.05±13.41$_{A}$
Texture	47.34±5.43	52.84±8.65	52.22±11.61	52.28±10.54
Mouth feeling	48.24±7.59	50.23±5.52	48.63±15.19	50.74±14.59
Overall Liking	48.24±7.74	53.36±14.58	24.26±16.34$_{AB}$	26.76±14.15$_{AB}$

The letter marked the difference between groups. Attributes marked with a letter denote that samples were significantly different (lower case P≤ 0.05, upper case P≤ 0.001).

The overall liking for white sauce with added low level of nanoemulsion (250mg /100g) and with added high level of bulk oil was decreased by 50% compared that of the control sample. Also, the white sauce sample with added high level bulk oil was found with stronger fishy aroma and fish taste, significantly higher compare to other samples. However, the white

sauce sample with added low level of bulk oil has a good acceptability, which is not significant from control. There are no statistically significant difference between the four samples were found when colour, salt taste, texture and mouth feeling were compared. Especially, the mouth feeling had similar score for the four samples. The white sauce added low level nanoemulsion sample has no significant differences on fish aroma and taste compare to control. However, the aftertaste and overall liking of white sauce added low level nanoemulsion was found to have significant differences compare to the control sample.

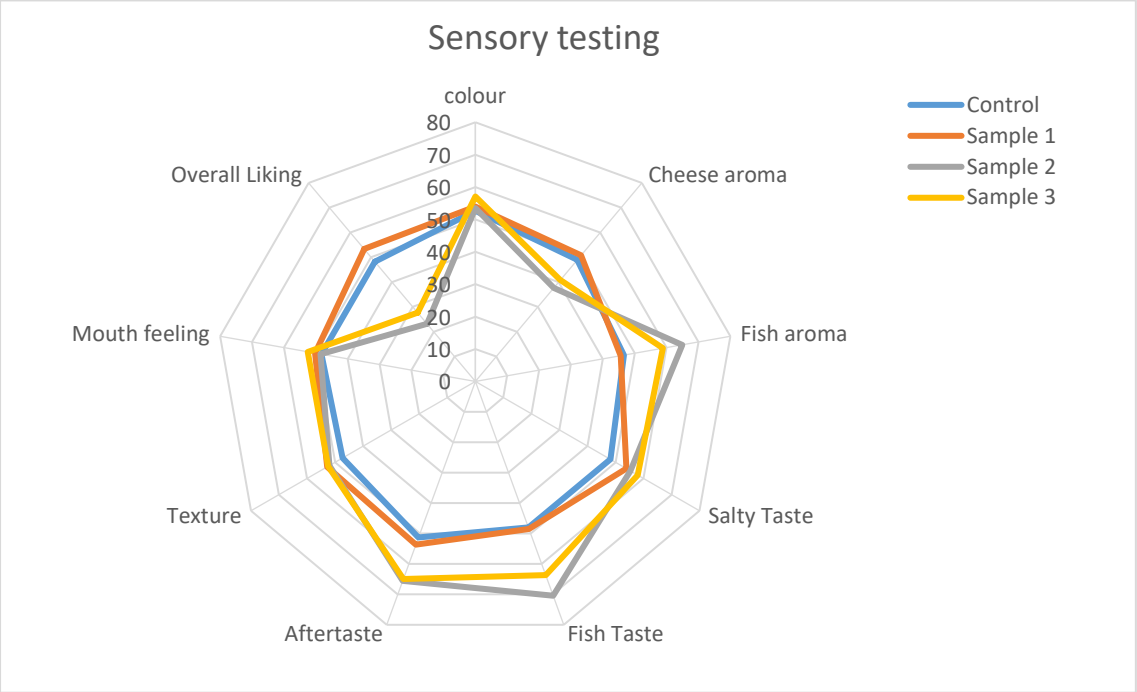

Figure 31 Sensory profile of white sauce with added algal oil and its nanoemulsion

6.4 Discussion

This study was conducted with white sauce co-operated with algal oil and its nanoemulsion. The white sauce has stronger cheese and butter aroma with creamy and saltiness taste, which may be able to cover the fishy smell and taste. The effect of added omega-3 oil nanoemulsion on appearance of white sauce samples in present study is same as the result of the previous study from Jamsshidi *et al*., (2018), in which the added microcapsules had no impact on the visual appearance of yogurt, and shows a regular and uniform distribution throughout during blending. Ghorbanzade, Jafari, Akhavan, and Hadavi (2017) also reported no significant differences between the control and fortified nano liposomal in terms of texture.

The sensory analysis to assess the sensory properties of yogurt enriched with omega-3 oil nanoemulsion was conducted by Lane (2014). The sensory testing results in Lane's research show a negative effect of added omega-3 oil nanoemulsion on the seven chosen attributes (appearance, aroma, flavour, texture, consistency, aftertaste and overall acceptability) when testing plain yogurt enriched with omega-3 oil nanoemulsion, and the comment on flavour included 'horrible', 'fishy' and 'sour'.

In this study, there is a significant difference in the fish taste of white sauce with added high level algal oil compare to control sample without any added, and the attributes of aftertaste and overall liking have significant differences compared to the control sample and added low level bulk oil sample. The white sauce added low level nanoemulsion sample has significant differences compare to the control sample on the aftertaste and overall liking.

The previous study (Doi, T., Wang, M., & McClements, D. J. (2018) found that the most unstable emulsion for coalescence was found the largest droplet size and tends to have better flavour retention. The observed difference in flavour retention is primarily due to differences

in the size and location of the oil droplets during cooking. In this study, high level algal oil and low level nanoemulsion were incorporated in white sauce without any flavoring agent, and had similar fisher flavour effect on aroma, taste and aftertaste. This results indicate that the emulsification of algal oil has a faster flavour release. In addition, these results also indicate that emulsified algal oil is above to enhance the intake of omega 3 fatty acids in human diet. However, further study is needed to mask the unfavorable flavor of algal oil.

6.5 Conclusion

The aim of the study was to investigate the effect of omega-3 nanoemulsion on its sensory properties of enriched food, in particular fat-related sensory attributes. It is interesting to observed that there was no or only very small differences in sensory perception between high level oil and low level nanoemulsion with selected emulsifiers, combination of lecithin and Tween 40. Sensory perception of fishy taste and aftertaste attributes was enhanced when the food samples incorporated nanoemulsion at same level DHA as bulk oil, indicating that the smaller droplet size can be affect the taste release in mouth. Interestingly, the sensory attribute 'mouth feeling' of white sauce had no significant changes with a low and high level of algal oil and its nanoemulsion. These results suggest that emulsified algal oil can be used to provide adequate DHA intake to the human diet. However, masking the unfavourable taste of algal oil is needed in their application.

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Chapter 7 Conclusions and further research

7.1 Conclusion

In this study, the omega-3 oil nanoemulsion was developed with selected emulsifiers and their combinations using ultrasonic processing. It was observed that the nanoemulsion has been stabilised with selected emulsifiers (LE/TW 90:10 LE/TW 70:30 & LE/TW 50:50), and the drop size of nanoemulsion decreased with an increase in the TW ratio of combined LE/TW. The smallest droplet size was achieved with the combination of lecithin and Tween 40 at a ratio of 50:50. It was also found that both the sodium alginate and gum Arabic were able to be coated on the droplets of nanoemulsion prepared.

In *in vitro* digestion testing, the omega-3 nanoemulsion developed with the LE/TW combination at ratio of 50:50 was found to be stable in gastric phase at pH 1.6, for the whole duration of 60 min where the drop size of nanoemulsion were at 267nm and around 50% drop size was less than 200 nm . However, droplet size of the nanoemulsions was not maintained in the duodenal phase at pH 6.8 during enzymatic hydrolysis. At the end of duodenal phase, a bigger portion of droplets in a range of 0.005 to 0.5 μm was found with digested nanoemulsion compared with the digested MIX. Furthermore, after centrifugation the aqueous phase of digested nanoemulsion sample had a droplets of 0.10 ± 0.002 μm which is much smaller than that from bulk oil MIX sample. The quantification of the aqueous phase DHA from digested samples by GC clearly the digestibility of nanoemulsion is much higher than the bulk oil sample and bulk oil emulsifier MIX sample.

In 5 weeks storage trial, the droplet size of nanoemulsions prepared with lecithin and Tween 40 only remained stable and there no significant changes regardless of the temperatures. However, the droplet size of nanoemulsion with combined lecithin and Tween 40 had

significant increases in droplet range measurements at higher temperatures after 5-weeks of storage. There were no significant differences in DHA fatty acid composition for all samples throughout the storage trial at all temperatures. The oxidation stability of tested samples in the storage trial was determined by five main volatile compounds, which were identified from tested samples, the results show the variation in oxidation stability of samples under different storage conditions. Nanoemulsion systems for both the sole and combined lecithin samples had more differences for development of volatile compounds at higher temperatures. This may be attributed to LA found in lecithin molecules being more unstable than DHA due to its position in the outer layer of lipid droplets and therefore closer to the aqueous phase of the nanoemulsion. In addition, the results demonstrated larger droplet size ranges did not offer a protective effect for the development of volatile compounds. Samples created using Tween 40 developed fewer volatile compounds than the lecithin containing samples.

Sensory testing show the white sauce with nanoemulsion have a stronger fishy taste and less overall liking than with bulk oil, indicating the smaller drop size more ready to spread and reach to sensors of mouth.

Overall, the results of current study are suggesting that emulsified algal oil is able to provide adequate DHA intake to the human diet. However, masking the unfavourable flavour of algal oil is needed in their application.

7.2 Future research and recommendation

In order to conduct the current study into further research, the bioavailability of nanoemulsion in human tail can be studied to related *in vitro* digestion model. The previous research from Lane *et al.* (2014) reported that LC3PUFA absorption from the nanoemulsion was significantly higher than the bulk oil from a randomised crossover trial. However, the

emulsion system used in the human trial was found unstable when the pH lower than 3.5, which lowed digestibility of DHA in *in vitro* testing as reported by Lin et al., (2014). The developed nanoemulsion system in current study was found stable in gastric phase with pH 1.6 and DHA in aqueous phase of digested nanoemulsion was much higher than bulk oil and emulsifier MIX, indicating that it could have better absorption than the others. Therefore, the human intervention trial would be able to provide on bioavailability of DHA of developed nanoemulsion.

In addition, based on the results obtained from this study, how lecithin affect the oxidation of nanoemulsion need a further investigation, which is able explain that the impact of structure and composition of emulsifiers on oxidation stability of emulsion.

How to mask the unfavourable flavour of foods with nanoemulsion is still a challenge, which can be a main task in the future research.

Appendixes

Appendix 1. 7th MMU Postgraduate Research Conference 2014 - Poster presentation

Appendix 2. NoWFOOD future food horizon in university of Chester Poster presentation

Appendix 3 Abstract of Faculty postgraduate research conference at university of Chester presentation

Appendix 4. Faculty postgraduate research conference at university of Chester Poster presentation

Appendix 5. Abstract of Publication

Appendix 6 Photographs of nanoemulsions stability by pH testing

Appendix 7. Droplets size measurement

Appendix 1. 7th MMU Postgraduate Research Conference 2014 - Poster presentation

Effect of omega-3 nano-emulsion stabilized with various emulsifiers on sensory properties of orange juice

QiQian ZHOU *, Dr Weili LI
*E-mail: qiqian.zhou@stu.mmu.ac.uk
Manchester Metropolitan University, Department of Food & Tourism Management, Manchester M15 6BH, United Kingdom

Introduction

The current delivery system of nutrients in the market could be further improved by using nano-emulsion technology, instead of just adding nutrients to enhance the nutritional properties.

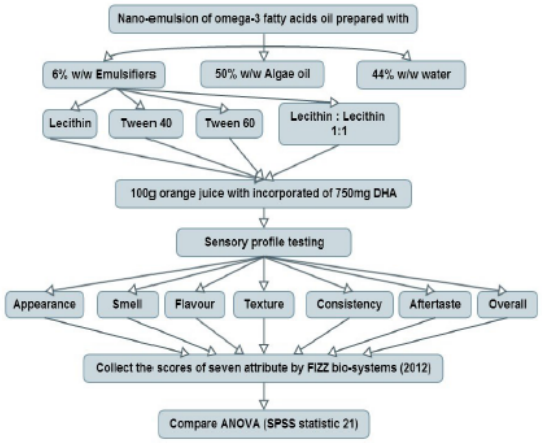
Omega-3 fatty acids are a set of polyunsaturated fatty acids, found in deep-sea fishes and certain plants that are beneficial to human health (Meyer *et al.*, 2003).

The previous studies showed that nano-emulsion of omega-3 fatty acids oil had significantly negative effects on sensory attributes of food (Lane, 2013), and demonstrated the sensory characteristics varied with emulsifiers and drop sizes of emulsions (Van Ruth *et al.*, 2002).

The aim for this work write the following:

•To evaluate the sensory profile and consumer acceptability of orange juice with the incorporation of omega-3 nano-emulsion stabilized by various emulsifiers, including lecithin, 50% lecithin plus 50% Tween 40, Tween 40 and Tween 60 by a sensory testing of 40 panellists.

Methods



Results & Discussion

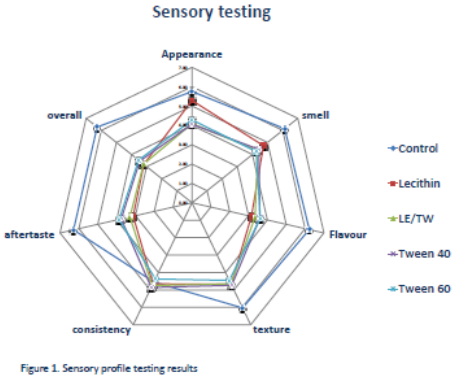


Figure 1. Sensory profile testing results

- The comparison between the four nano-emulsion samples had no significantly difference on the six attributes

- The attributes of orange juice enrich omega-3 nano-emulsion samples on smell, flavour, aftertaste and overall acceptability were statistically significance lower than the control sample (P≤ 0.001).
- The overall acceptability of nano-emulsion samples had a large reduced almost 50% scores form control sample.
- There are no statistically significant difference were found between five samples when consistency were compared.
- The nano-emulsion with lecithin was better than the other three nano-emulsion sample and rated closest to the control sample

Sample	Control		LE		LE/TW		TW40		TW60	
Attribute	Mean	SD.	Mean	SD.	Mean	SD.	Mean	SD.	Mean	SD.
Appearance***	5.75	1.77	5.29	1.48	4.09	1.64	4.06	1.61	4.28	2.05
Smell***	6.14_A	1.46	4.73_B	1.61	4.35_B	1.72	4.43_B	1.4	4.24_B	1.79
Flavour**	6.22_A	1.95	3.15_B	2.23	3.35_B	2.24	3.6_B	2.05	3.63_B	2.46
Texture***	6.03	1.45	4.67	1.83	4.65	1.7	4.74	1.59	4.45	1.78
Consistency***	4.54	1.45	4.65	1.78	4.75	1.62	4.86	1.52	4.37	1.78
Aftertaste**	6.29_A	1.83	3.12_B	2.09	3.29_B	2.16	3.76_B	2.07	3.86_B	2.35
Overall**	6.28_A	1.92	3.19_B	2.12	3.2_B	2.17	3.46_B	1.95	3.53_B	2.23

Table 1. Statistical analysis of sensory profile testing
Key: 1. Different letters in the same row denote results that are significantly different to one another.
2. Attributes marked with asterisk denote that samples were significantly different(** P≤ 0.01, *** P≤ 0.001).

Conclusions

- The orange samples for the sensory profile testing contained 750mg DHA per 100g orange juice sample, to achieved the level of 200-1000mg omega-3 daily intake for health improve.
- The omega-3 nano-emulsions with emulsifiers has a significant negative effect on the appearance, smell, flavour, texture, aftertaste and overall acceptability of the orange juice product.
- Further improvement of the omega-3 nano-emulsion sensory profile are recommended to focus on the attributes of smell, flavour and aftertaste through the nano-emulsion technology.

Acknowledgements

I would like to thank my supervisors Dr Weili Li, Dr Kritika Mahadevan, technician Carolyn Wilson and Anika Ng for their help with sensory analysis.

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Appendix 2. NoWFOOD future food horizon in university of Chester Poster presentation

Omega-3 enriched Nano-emulsion delivery system for food application

QiQian, ZHOU, and Weili LI
Health research centre. Food, Nutrition and Health research group
Department of Food and Tourism Management
Manchester Metropolitan University, Manchester M15 6BH

Introduction

- Omega-3 is a set of polyunsaturated fatty acids, found in deep-sea fishes and certain plants that are beneficial to human health (Meyer *et al*., 2003).
- The previous studies showed that nano-emulsion omega-3 oil improved the bioavailability of omega-3 fatty acids (Lane et al., 2014).
- However, it was observed this system was not stable under highly acidic gastric conditions (pH=1.6) (Lin, et al., 2014).

The aims of current study are to develop a new nano-emulsion delivery system for Omega-3 oil, which will be stable in the *In vitro* digestion model.

Methods

- Nano-emulsion of omega-3 fatty acids oil was developed with selected 6%w/w emulsifiers, including lecithin and Tween 40, 50% (w/w) algae oil (Life DHA™ S35-O300, DSM Ltd., Columbia) and 44% (w/w) water and was processed in a homogenizer (Silverson Machine Ltd, England) and a UP400S Ultrasonic processor(Hielscher, Germany) by following the method developed by Lane et al., (2013).
- The nano-emulsion system was digested *in vitro* digestion model, which is a useful alternative to human models (Lin et al., 2014).
- The droplet sizes of omega-3 nano-emulsion prepared and digestive fluid were determined on Malvern Mastersizer 2000 (Malvern Instruments Ltd, UK).

Results & Discussion

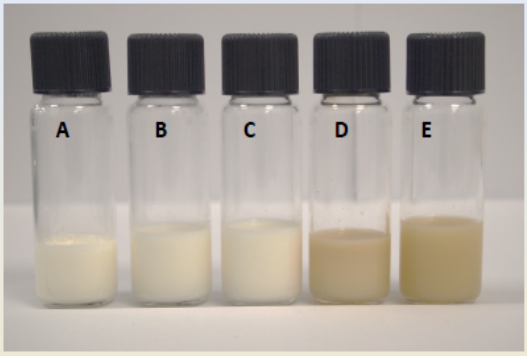

Figure 1. The photos of omega 3 nano-emulsion and digested fluids of *in vitro* digestion model . A. Omega 3 nano-emulsion; B. Digested fluid of pH 1.6 gastric phase (2 min); C. Digested fluid of pH 1.6 gastric phase (60 min); D. Digeted fluid of duodenal phase (5 min); E. Digested fluid of duodenal phase (60 min) .

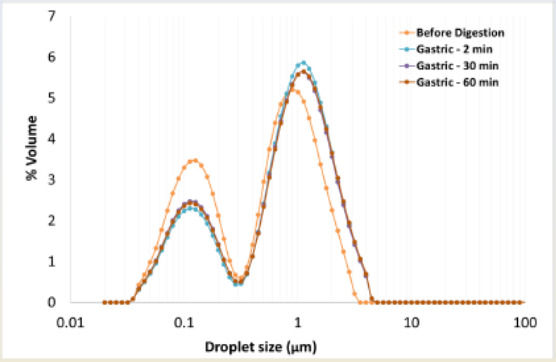

Figure 2. The oil droplet size distribution of omega 3 nanoemulsion and digested fluids with 2, 30 and 60 min digestion time at pH 1.6 gastric phase

- The results in Fig. 1 show that there was no clear phase separation in the current digestion study, which includes 60 min digestion at pH 1.6 gastric phase and 60 min digestion in duodenal phase. It is obvious that the developed omega-3 nano-emulsion delivery system was much more stable under the digestion conditions compared with one used in the previous study (Lin et al., 2014)

- The results in Fig 2 present that droplets of developed omega-3 oil nano-emulsion delivery system were distributed in a range of 0.1 to 1 µm and there were very limited increase in droplet size under the pH 1.6 *in vitro* digestion model.
- The average oil droplet diameter for emulsion is ($D_{3,2}$) is 232 nm and for digestied fluid at 5, 30 and 60 min digestion time are respectively.

Conclusions

- The developed omega-3 nano-emulsion prepared with selective emulsifiers was stabilized in the *in vitro* digestion testing under highly acidic gastric conditions (pH=1.6).
- This stable nano-emulsion will potentially further enhance the bioavailability of omega 3 fatty acids.

References

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Lane, K., Li, W., Smith, C. & Derbyshire, E. (2013). 'The bioavailability of an omega-3-rich algal oil is improved by nanoemulsion technology using yogurt as a food vehicle. International Journal of Food Science and Technology.
Lin, X., Wang, Q., Li, W. & Wright, A. J. (2014) '*In vitro* digestive stability, lipolysis and bioaccessibility of an algal oil-containing lecithin-stabilized emulsion' Poster Presented at the AOCS Annual Meeting &Expo, San Antonio, Texas.

Acknowledgements

We would like to acknowlege Professor Saphwan Al-Assaf from Glyndŵr University for his kindly support in the droplet size testing in the current study.

Appendix 3 Abstract of Faculty postgraduate research conference at university of Chester presentation

Improved omega-3 enriched nano-emulsion *In vitro* digestibility

Abstract

QiQian ZHOU and Prof. Weili LI

The current delivery system of nutrients in the market could be further improved by using nano-emulsion technology instead of just simply enriching with micronutrients to enhance the nutritional properties. Omega-3 fatty acids are a set of polyunsaturated fatty acids, found in deep-sea fishes and certain plants that are beneficial to human health. The previous studies showed that nano-emulsion using 100% lecithin as emulsifier could not stabilized during the *In vitro* digestion model (Lin et al, 2014). This study aimed to develop the stability and digestibility of omega-3 nano-emulsion with different combination emulsifiers. Omega-3 nano-emulsion was prepared with selected emulsifiers, Lecithin and equal ratio of combined Tween40 and lecithin, with Algae oil and water used homogenizer and ultrasound. The *In vitro* digestion experiments simulating a fed state gastric and duodenal digestion using methods by Lin et al (2014). The droplet size measurement of nano-emulsion samples before, during and after digestion were determined by the Mastersizer 3000 laser light-scattering analyzer (Malvern Instrument Ltd, UK). The fatty acids were extracted from digested samples and analysis using a GC Clarus 480 system (PerkinElmer Inc, USA). The results shown that the omega-3 nano-emulsion (LE/TW 5:5) were stabilized during the digestion gastric phase for 60 mines, compare to the results of omega-3 nano-emulsion (LE 100%) at the gastric phase for 60 mines, which the particle size diameter has significantly different ($P < 0.05$). The remaining DHA content from the digested nano-emulsion was quantified, which was 47.34 mg/ml; and in comparison to the DHA content remained in the digested oil sample, it was much lower at 16.53 mg/ml which has a significant difference ($P<0.05$). In conclusion, combined emulsifiers have a great impact on the stability of nano-emulsiom during the *in vitro* digestion. In addition, Omege-3 enriched nano-emulsion has high digestibility and bioavailability than the Algae oil.

Appendix 4 Faculty postgraduate research conference at university of Chester Poster presentation

The oxidation study on long chain omega-3 polyunsaturated fatty acid oil and Nanoemulsions

Qiqian Zhou[1], Katie. E. Lane[2] and Weili Li[1]

1 Department of clinical sciences and nutrition, the University of Chester
2 School of Sport Studies, Leisure and Nutrition, I.M. Marsh Campus, Liverpool

Background and Objective

▪ Algal oil is rich in docosahexaenoic acid (DHA) which has cardiovascular benefits[1].

▪The long chain omega-3 (n-3) polyunsaturated fatty acids (LC3PUFA) in human diets are mainly derived from oily fish, fish oil or fish oil based supplements1.

▪At present, the consumption of oil fish in the UK is far below the recommended level2. LC3PUFA's non-fish sources such as algae oil are particularly important for vegetarians/vegetarians, non-fish eaters, and pregnant women3. Algal oil has recently become a sustainable vegetarian source of long chain omega 3 fatty acids long chain omega 3 fatty acids4.

▪In previous work, high DHA vegetative algal oil load of 50% (w / w) was successfully used to establish an oil-in-water nanoemulsion system suitable for functional food enrichment5. Nanoemulsions systems having a droplet size in the range of 20-500 nm that can improve LC3PUFA bioavailability through ultrasound processing6. However, the application of ultrasound may also affect the oxidation stability of LC3PUFA7.

The aim of this study was to analyse the oxidative stability of nanoemulsions containing algae oils rich in docosahexaenoic acid (22:6n-3; DHA), and with a bulk oil and a crude emulsion with the same composition Ranges of temperatures using gas chromatography headspace analysis (GCHS), gas chromatography (GC) and droplet size analysis.

Materials and Methods

▪ **Meal samples:** The emulsions are method with selected 6% (w/w) of emulsifiers i.e. lecithin (LN), Tween 40 (TN), and equal ratio Tween 40 and lecithin (LTN), with 50% (w/w) of algal oil and 44% (w/w) of water. The sample of nano-emulsion was processed under an ultrasonic processor (BSP-1200 Ultrasonic processor, USA).

▪ **The storage trial for algal oil and algal oil emulsion:** The algal oil and algal oil nanoemulsions were stored in incubators set at 4ºC, 20ºC and 40ºC for 5 weeks. The droplet sizes and volatile compounds were determined at baseline, week 1, week, 2 and week 5. Fatty acid composition was analysed at the beginning and the end of storage trial.

▪ **Particle size:** The droplet size measurement of emulsion samples since week 1 to week 6 at 4, 20, 40 degrees were determined by the Mastersizer 3000 laser light-scattering analyzer (Malvern Instrument Ltd, UK) with a small sample dispersion unit set 2400 rpm. Samples were dispersed in distill water (refractive index=1.488, 25˚C).

▪ **Gas chromatography Headspace (GCHS):** Samples of 2g nanoemulsion were prepared with 1 ml 1% NaCl solution, added to HS vial and vortexed for 30 secs. The samples were heated in a Headspace sampler (TuborMatrix 40 PerkinElmer Inc, USA) at 100°C for 60 min and injected under the conditions. The samples were analysed by HS GC Clarus 580, (PerkinElmer Inc, USA) equipped with a Flame Ionization Detector. The volatile compounds were identified by reference to the retention time of standards. Analysis was performed in triplicate on individual vials for each time point.

▪ **Gas chromatography (GC):** The fatty acid composition analysis of algal oil and nanoemulsion samples was performed by following a method developed from FAME analysis method by NoWFood research centre. Analysis was performed in triplicate on individual vials for each time point.

▪ **Replication and statistics:** Measurements were performed in triplicate. Results were analyzed with two-way ANOVA using SPSS 24.0 software and Bonferroni post hoc testing. Significance was considered at $p \leq 0.05$.

Results

A. Emulsions Droplet size

▪ Average diameter of LTN emulsion droplets ($D_{3,2}$) = 0.17 ± 0.003 µm (Fig.1).
▪ Average diameter of TN emulsion droplets ($D_{3,2}$) = 0.24± 0.002 µm (Fig.1).
▪ Average diameter of LN emulsion droplets ($D_{3,2}$) = 0.34 ± 0.001 µm (Fig.1).

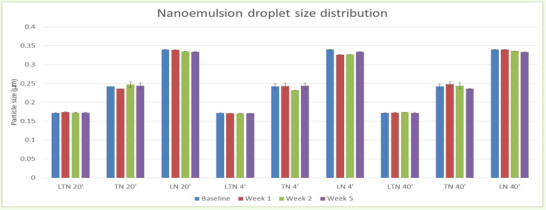

Figure 1. Droplet size distribution of nanoemulsion stabilized with different emulsifier of Lecithin, Tween 40 and Tween40/Lecithin storage at 4, 20 and 40°C in 5 weeks.

▪Increased temperature and storage periods had no significant effect on the droplet size of all samples stored at 4, 20 and 40 ºC ($p \geq 0.05$).

B. Oil and nanoemulsions oxidation testing by GC-HS

Table 1. GC-HS analysis on oxidizsed Propanal produced by algal oil and nanoemulsions during storage at 4, 20 and 40°C in 5 weeks.

ºC	Week	0		1		2		5	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
40	Oil	24842	12532	-	-	119512	27131	266506^{a,A}	98586
	LN	44913	2201	306664^{a,A}	9482	203925^{a,A}	90123	263618^{a,A}	67145
	TN	13628	7430	107139	46935	357994^{a}	85487	299928^{a,A}	16948
	LTN	32084	0.00	71597	9864	271886^{a}	42527	458613^{a,A}	17751
20	Oil	24842	12532	-	-	153570^{a,A}	57352	87246	3926
	LN	44913	2201	165865^{a}	489	125430	44876	175276^{a}	46031
	TN	13628	7430	56054	33169	226867^{a}	3241	196127^{a}	32536
	LTN	32084	0.00	57090	2770	67144	77958	209987^{a}	123433
4	Oil	24842	12532	-	-	46504	32846	7065	5153
	LN	44913	2201	102757	38255	150174	6087	110398	38228
	TN	13628	7430	59698	5399	161155	135887	161859	12819
	LTN	32084	0.00	59321	773	140201	14239	130062	11497

a Significantly different at p≤0.05 compared with week 0 at the same storage temperature;
A Significantly different at p≤0.05 compared with 4 ºC at the same storage time.

▪Increased temperature and storage periods had a significant effect on the development of propanal for all samples stored at 40 ºC ($p \leq 0.05$).

▪Nanoemulsions prepared with lecithin alone had significantly higher development of propanal in week 1 at 40 and 20 ºC respectively ($p \leq 0.05$).

▪There were no significant differences for emulsion/emulsifier type for samples stored at 4 ºC.

Results

C. Fatty acid composition of the algal oil and nanoemulsions

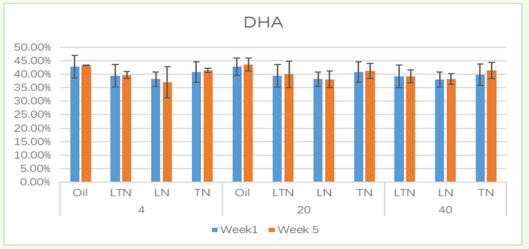

Figure 2. Fatty acid composition of oil and nanoemulsions storage at 4, 20 and 40°C in 5 weeks.

▪ There was no significant differences on DHA composition results within the weeks and temperature ($p \geq 0.05$).

Conclusions

▪Overall, storage periods and temperature had no influence on droplet size of nanoemulsions, and DHA fatty acid composition of oil and nanoemulsions.

▪The increased temperature and storage period had effect on development of propanol of all samples when stored at 40 ºC.

▪Nanoemulsion with lecithin alone had higher influence on development of propanal at first week.

▪To further evaluate oxidation status, research should now be conducted over the same storage periods and temperature to identify and measure other recognised volatile compounds.

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Appendix 5. Abstract of Publication

Elsevier Editorial System(tm) for Journal of Functional Foods Manuscript Draft

Manuscript Number: JFF-D-19-00309

Title: The composition and oxidative stability of vegetarian long chain omega-3 polyunsaturated fatty acid algal oil nanoemulsions suitable for functional food enrichment

Article Type: Full Length Article

Keywords: Omega-3 fatty acids; Algal oil; Nanoemulsion; Oxidative stability; Lecithin; Tween 40

Corresponding Author: Dr. Katie Elizabeth Lane, Ph.D

Corresponding Author's Institution: Liverpool John Moores University

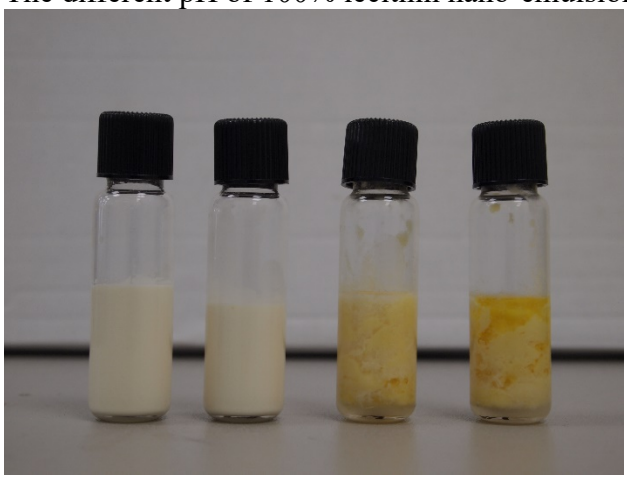
First Author: Katie Elizabeth Lane, Ph.D

Order of Authors: Katie Elizabeth Lane, Ph.D; Qiqian Zhou, MPhil; Sharon Robinson, PhD; Weili Li, PhD

Abstract: Influences of storage time, temperature and emulsifier on the oxidative stability of algal oil-in-water nanoemulsions created by ultrasound using lecithin and/or Tween 40 were examined. Nanoemulsions and bulk oil were stored at 4, 20 and 40°C for 5-weeks. Fatty acid composition, droplet size ranges and volatile compounds were analysed. Fatty acid composition remained stable with no significant differences for temperature and storage. There were no significant DHA losses whencomparing sample storage time and temperature. Emulsion droplet sizes remained stable with no significant differences for storage. Droplet size ranges were significantly larger for lecithin only samples throughout the study duration. Analysis of volatiles identified five oxidised compounds and showed all samples had lowest levels when stored at 4°C. Lecithin samples (sole and combined) had significantly increased volatiles at higher temperatures which may be due to instability of linoleic acid in lecithin molecules at the outer layers of oil droplets.

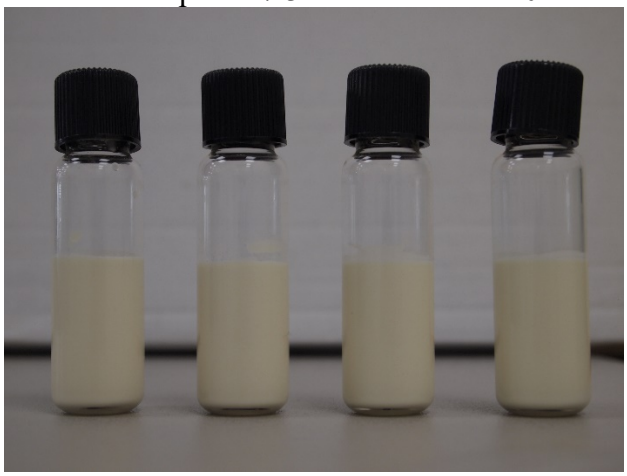
Appendix 6 Photographs of nanoemulsions stability by pH testing

The different pH of 100% lecithin nano-emulsion



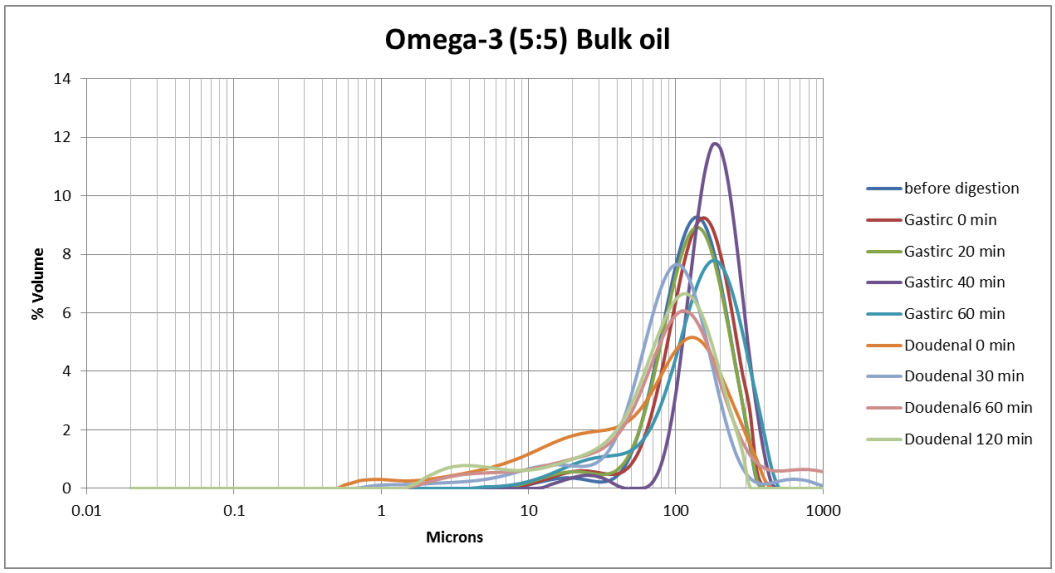
The different pH of 9:1 lecithin/Tween 40 nano-emulsion

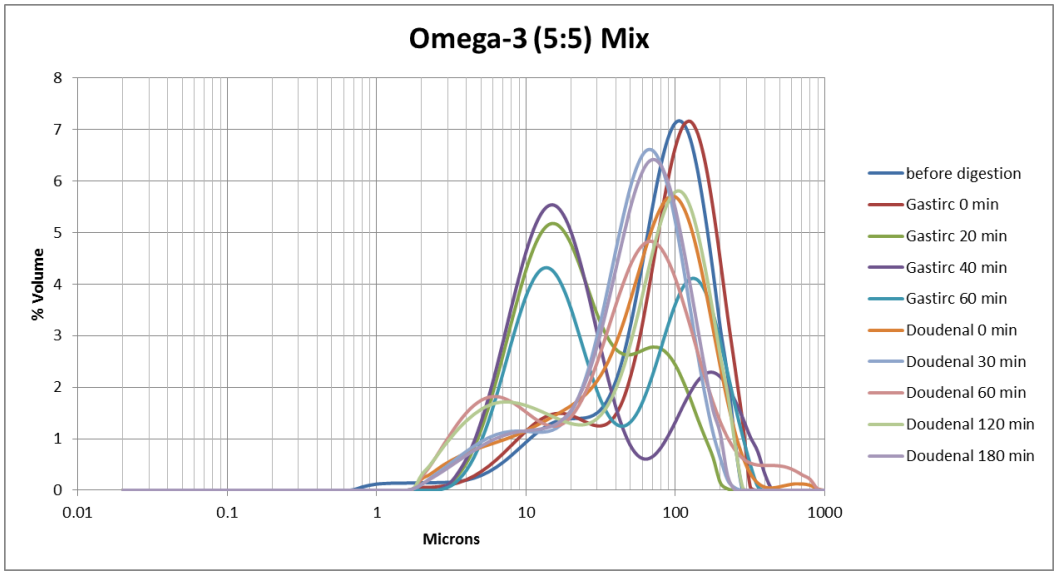
The different pH of 7:3 lecithin/Tween 40 nano-emulsion

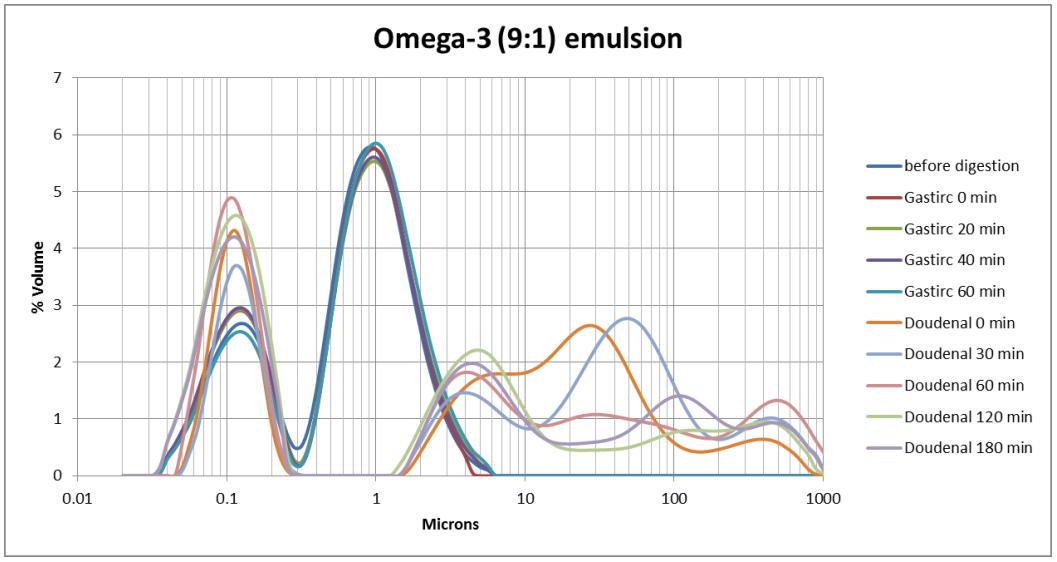


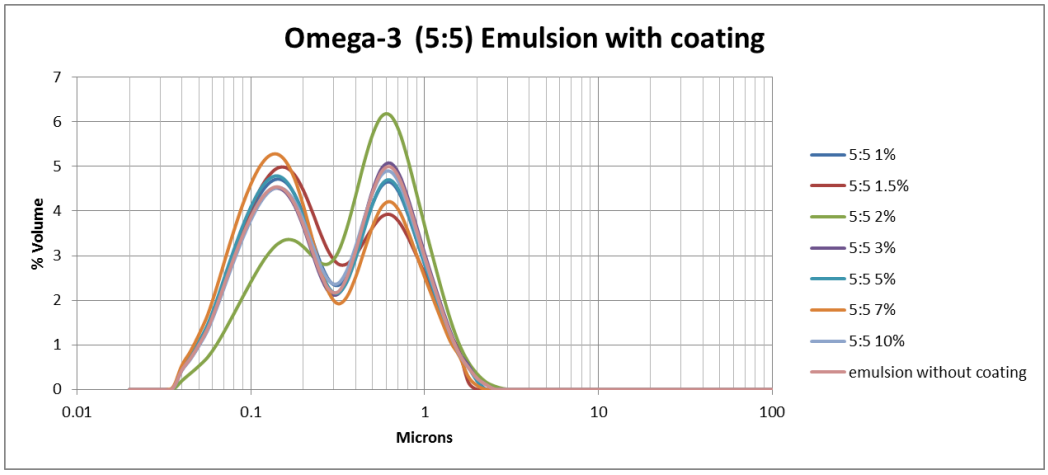
The different pH of 5:5 lecithin/Tween 40 nano-emulsion

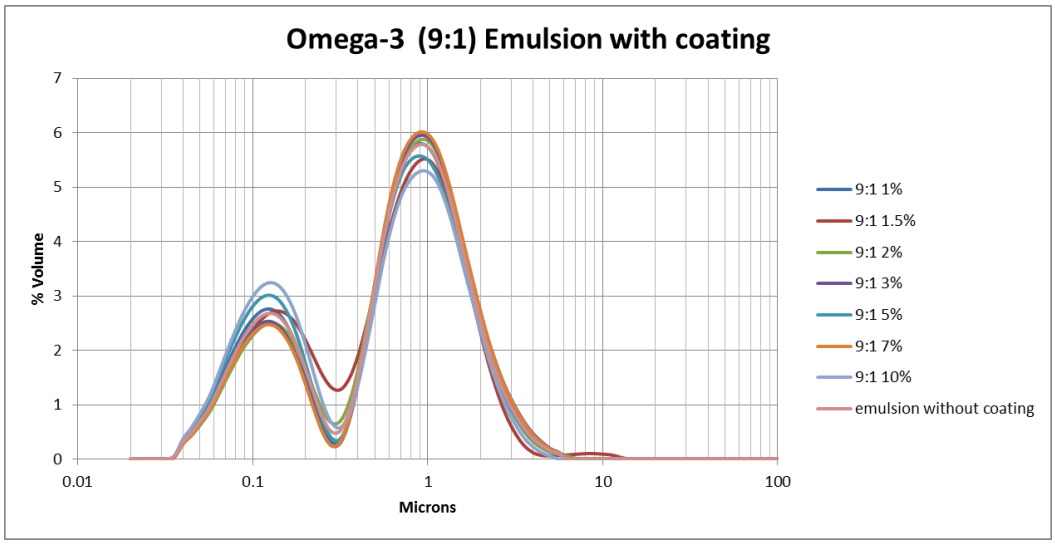
Appendix 7. Droplets size measurement








Omega-3 (5:5) Emulsion with coating
% Volume
Microns
0.01
0.1
1
10
100
0
1
2
3
4
5
6
7
5:5 1%
5:5 1.5%
5:5 2%
5:5 3%
5:5 5%
5:5 7%
5:5 10%
emulsion without coating


Omega-3 (9:1) Emulsion with coating
% Volume
Microns
0.01
0.1
1
10
100
0
1
2
3
4
5
6
7
9:1 1%
9:1 1.5%
9:1 2%
9:1 3%
9:1 5%
9:1 7%
9:1 10%
emulsion without coating